Activation Profiles and Regulatory Cascades of the Human Kallikrein-related Peptidases*[S]

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The human kallikrein (KLK)-related peptidases are the largest family of serine peptidases, comprising 15 members (KLK1–15) and with the majority (KLK4–15) being identified only within the last decade. Members of this family are associated with important diseased states (including cancer, inflammation, and neurodegeneration) and have been utilized or proposed as clinically important biomarkers or therapeutic targets of interest. All human KLKs are synthesized as prepro-forms that are proteolytically processed to secreted pro-forms via the removal of an amino-terminal secretion signal peptide. The secreted inactive pro-KLKs are then activated extracellularly to mature peptidases by specific proteolytic release of their amino-terminal propeptide. Although a key step in the regulation of KLK function, details regarding the activation of the human pro-KLKs (i.e. the KLK "activome") are unknown, to a significant extent, but have been postulated to involve "activation cascades" with other KLKs and endopeptidases. To characterize more completely the KLK activome, we have expressed from Escherichia coli individual KLK propeptides fused to the amino terminus of a soluble carrier protein. The ability of 12 different mature KLKs to process the 15 different pro-KLK peptide sequences has been determined. Various autolytic and cross-activation relationships identified using this system have subsequently been characterized using recombinant pro-KLK proteins. The results demonstrate the potential for extensive KLK activation cascades and, when combined with available data for the tissue-specific expression of the KLK family, permit the construction of specific regulatory cascades. One such tissue-specific cascade is proposed for the central nervous system.

The kallikrein (KLK)[3]-related peptidases (1) are a multigene group of the chymotrypsin-like S1A family of clan PA(S) serine peptidases (2), co-localized to chromosome locus 19q13.4 (positioned between the testicular acid phosphatase and *Siglec* gene families). The 15 KLK members (KLK1–15) represent the largest cluster of serine peptidases within the human genome (3). The KLK genes exhibit a high degree of identity, having five coding exons of similar size and with conserved intron phases.

Many of the KLKs are under the control of steroid hormones, and several KLKs are differentially regulated in specific types of cancer (4–7). However, the physiological consequence of such regulation is not well understood; nonetheless, the KLKs contain important known, and proposed, cancer biomarkers (8). KLK3 ("prostate-specific antigen") is a widely used cancer biomarker for prostate cancer screening; levels greater than 10 ng/ml of blood serum prostate-specific antigen positively correlate with prostate cancer (9–11). Recent studies indicate the presence of elevated levels of KLK2 in addition to KLK3 in malignant prostatic tissue and serum; so the ratio of KLK2 to KLK3 is emerging as an additional important cancer diagnostic and prognostic tool (12–14). KLK5, -7 and -14 are co-expressed in skin and have been postulated to function in the proteolytic events associated with skin desquamation and disorders thereof (15, 16). KLK6 has been shown to be involved in the process of inflammatory demyelination, and its specific inhibition can delay the onset and reduce the severity of animal models of such disease (17). These and other observations identify the KLK family as gaining importance in the diagnosis and treatment of serious human diseases.

All KLK proteins are naturally synthesized as prepro-enzymes that are proteolytically processed upon entering the secretory pathway to release the pre-peptide, yielding the secreted pro-form. The secreted pro-form is subsequently converted into the enzymatically active mature form by proteolytic release of the amino-terminal propeptide. Abnormal functional levels of the KLKs in diseased states may be influenced by specific activation or inhibition events. The familiar regulatory cascades of peptidases involved in thrombogenesis, fibrinolysis, and the complement system suggest that the KLK family may similarly participate in cascades of activation that regulate their

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[3] The abbreviations used are: KLK, kallikrein; FGF, fibroblast growth factor-1; PBS, phosphate-buffered saline; EK, enterokinase; DTNB, 5,5′-dithio-bis(2-nitrobenzoic) acid; AMC, 7-amino-4-methyl coumarin; CNS, central nervous system; Boc, *t*-butoxycarbonyl; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

FIGURE 1. **The amino acid sequence (*single letter code*) for each of the pro-KLK-(1–15) amino termini.** The residues comprising the native KLK pro-sequences are indicated in *boldface letters* and are numbered according to the standard nomenclature (75) where cleavage between the P1 and P1′ positions (indicated by *arrow*) results in activation of the KLK. The pro-KLK4 sequence was altered at the P3 position to substitute the native Cys residue by Ser (indicated by *asterisk*), so as to avoid disulfide bond formation.

	His-tag/Linker	P7	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′	P6′	Fusion
pro-KLK1	MHHHHHHAS-	A	P	P	I	Q	S	R	I	V	G	G	W	E	-EF(FGF)
pro-KLK2	MHHHHHHAS-	V	P	L	I	Q	S	R	I	V	G	G	W	E	-EF(FGF)
pro-KLK3	MHHHHHHAS-	A	P	L	I	L	S	R	I	V	G	G	W	E	-EF(FGF)
pro-KLK4	MHHHHHHASVSG-	-	-	-	S	S*	S	Q	I	N	G	E	D		-EF(FGF)
pro-KLK5	MHHHHHHAS-	S	D	D	S	S	S	R	I	I	N	G	S	D	-EF(FGF)
pro-KLK6	MHHHHHHASWA-	-	-	E	E	Q	N	K	L	V	H	G	G	P	-EF(FGF)
pro-KLK7	MHHHHHHAS-	E	E	A	Q	G	D	K	I	I	D	G	A	P	-EF(FGF)
pro-KLK8	MHHHHHHASSA-	-	-	-	Q	E	D	K	V	L	G	G	H	E	-EF(FGF)
pro-KLK9	MHHHHHHASHGWA-		-	-	-	D	T	R	A	I	G	A	E	E	-EF(FGF)
pro-KLK10	MHHHHHHAS-	L	P	Q	N	D	T	R	L	D	P	E	A	Y	-EF(FGF)
pro-KLK11	MHHHHHHASGG-	-	-	-	-	E	T	R	I	I	K	G	F	E	-EF(FGF)
pro-KLK12	MHHHHHHASSQA-	-	-	-	A	T	P	K	I	F	N	G	T	E	-EF(FGF)
pro-KLK13	MHHHHHHASVS-	-	-	Q	E	S	S	K	V	L	N	T	N	G	-EF(FGF)
pro-KLK14	MHHHHHHAS-	S	Q	E	D	E	N	K	I	I	G	G	H	T	-EF(FGF)
pro-KLK15	MHHHHHHASA-	-	-	Q	D	G	D	K	L	L	E	G	D	E	-EF(FGF)

pro-KLK5 native extended propeptide: DHPSNTVPSGSNQDLGAGEDAR-

function (15, 18–21); it is therefore of substantial interest to characterize the potential for KLK activation cascades (*i.e.* the KLK activome).

A comparison of the primary structure of the KLKs indicates that 11 members (KLK1, 2, 4–6, 8, 10–14) contain an Asp residue at the base of the S1 pocket (at position 189 using the chymotrypsin numbering scheme) and are therefore expected to exhibit trypsin-like specificity (22). The remaining members (KLK3, -7, -9, and -15) are expected to exhibit chymotrypsin-like, or novel, specificities. Reports of the substrate specificities of the KLK family include examples of trypsin-like, chymotrypsin-like, or dual substrate specificities (15, 23–29), in general agreement with the nature of residue 189. Regarding the KLK propeptide sequences (Fig. 1), activation requires specific hydrolysis after Arg (KLK1–3, -5, and -9–11), Lys (KLK6–8 and -12–15), or Gln (KLK4). Within the trypsin-like group of KLKs, examples of preferential specificity for hydrolysis after Arg *versus* Lys residues have been reported (28–30). Thus, members of the KLK family that exhibit specificity for Arg, but have a P1 Lys in their pro-sequence, may be incapable of autoactivation (31); similarly, KLKs with chymotrypsin-like specificity that have either Arg or Lys in the P1 position of their propeptide may be incapable of autoactivation . In both of these cases, a distinct protease would regulate activation.

The study of peptidase activation can present with various technical challenges. In the case of the KLKs the isolation of mature or pro-KLK from natural tissues is not practical, and commercially available mature and pro-forms of the KLKs currently represent a subset of the total 15-member family. In principle, it is a straightforward matter to produce recombinant pro-KLK proteins; however, in practice, this approach can be complicated by activation or degradation because of the action of endogenous host peptidases (30, 32, 33). Furthermore, activation studies can be complicated if the mature KLK is capable of autolytic inactivation (28, 29) or can attack and inactivate the activating peptidase. In an effort to provide rapid lead information regarding details of the KLK activome, and to circumvent the above technical concerns, we have taken the approach of characterizing the hydrolysis of the KLK propeptides as fusion proteins. Propeptide fusion constructs have been successfully utilized to study activation or release of fused heterologous

proteins via the action of specific peptidases (34, 35) and indeed form the basis of highly useful recognition cleavage sites (*e.g.* the enterokinase recognition sequence) for the release of affinity-tagged heterologously expressed recombinant proteins.

We report the hydrolysis profile for all 15 pro-KLK fusion protein substrates with 12 different mature KLKs. The results demonstrate the potential for extensive activation interactions between members of the KLK family. Several KLKs demonstrate the potential for autoactivation; among these, three are known from the literature, and others are novel. A number of potential cross-activating relationships between different KLKs are also identified, including several reciprocal cross-activation relationships that can result in geometric amplification of an initial activation event. Eleven of the activation relationships identified from this study are subsequently confirmed utilizing recombinant pro-KLK proteins. Combining the activation information with available data regarding the tissue-specific distribution of the KLK permits construction of hypothetical tissue-specific activation cascades, and one such cascade for the CNS is proposed.

EXPERIMENTAL PROCEDURES

Our design for an appropriate fusion protein utilized the available x-ray structure data for human pro-KLK6 (32), which shows that the amino-terminal KLK propeptide is highly solvent-accessible and unstructured (Fig. 2). The human fibroblast growth factor-1 (FGF) protein exhibits similar structural features of its amino terminus, the first 10 residues of which are oriented toward solvent and are unstructured in both x-ray and NMR data (36, 37). We have previously studied a mutant form of FGF that exhibits enhanced properties of thermostability and solubility (38). In addition, this FGF mutant has two surface Arg residues deleted, which render the mutant generally resistant to internal tryptic cleavage. In initial enzymatic digests with mature KLK6 (a trypsin-like KLK) and KLK3 (a chymotrypsin-like KLK), this mutant FGF protein exhibited no detectable internal hydrolysis after extended incubation (data not shown); thus, it appeared to be an ideal candidate as a benign, soluble, carrier protein for pro-KLK peptide fusion constructs. The size of the intact pro-KLK fusion protein (~16,700 Da) in comparison with the size of the released KLK propeptide region (~1,900 Da), or carboxyl-terminal FGF fragment (~14,800 Da), permits rapid quantitation of propeptide hydrolysis using SDS-PAGE (with confirmation provided by mass spectrometry). Because the released FGF "product" is not an active peptidase, complications associated with secondary proteolytic events are avoided.

As mentioned above, the KLKs belong to the S1A chymotrypsin-like family of serine peptidases. In the chymotrypsin family of peptidases, the key interactions affecting specificity
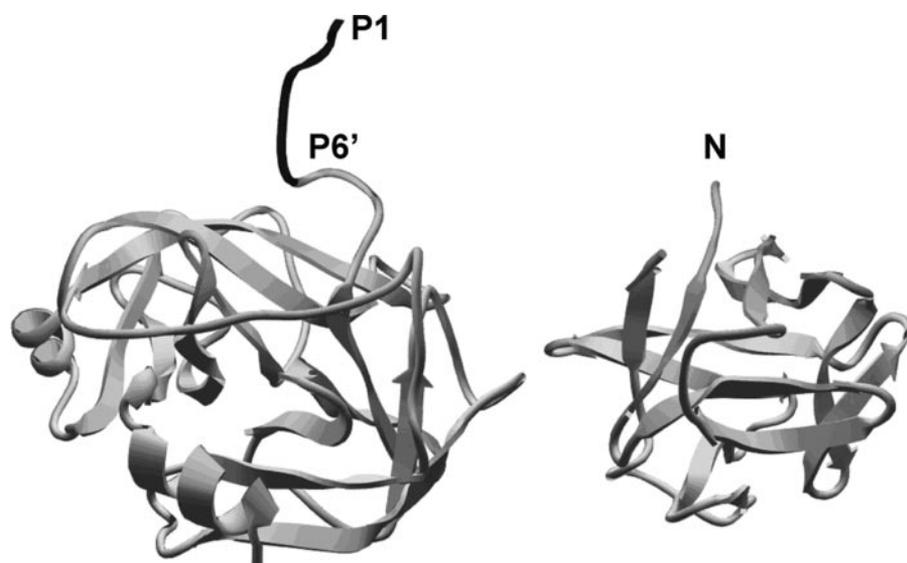
FIGURE 2. *Left panel,* a ribbon diagram of human pro-KLK6 (Protein Data Bank code 1GVL) with the P1 through P6′ positions of the amino terminus propeptide indicated by *black shading* (the P7 through P2 propeptide residues are undefined in the x-ray structure). *Right panel,* a ribbon diagram of human FGF (Protein Data Bank code 2AFG) with the amino terminus indicated. The amino terminus in this structure is residue position 11, and positions 1–10 are unstructured (both proteins are depicted using the same scale).

(GCTAGC) and EcoRI (GAATTC) restriction sites were inserted between the amino-terminal His tag and amino acid position 10 of the FGF gene by PCR mutagenesis (oligonucleotides purchased from Integrated DNA Technologies, Coralville, IA). These restriction sites are not found in any of the KLK sequences, or the FGF gene, and therefore permit easy insertion of DNA "cassettes" corresponding to pro-KLK sequences. This modified FGF construct was inserted into the pET21a vector (Novagen, Madison, WI), and the correct nucleotide sequence was confirmed. The vector was subsequently digested with NheI and EcoRI restriction enzymes and gel-purified ("vector fragment"). Oligonucleotides (coding and noncoding) were synthesized and hybridized to produce duplex DNA fragments ("insert fragments") corresponding to the prosequences of KLK1–15 with appropriate NheI and EcoRI halfsites. The KLK pro-sequences included in the fusion construct spanned the complete set of pro-KLK P positions, through the P6′ position, in each case (Fig. 1) Some KLKs contain relatively short propeptides (*e.g.* the KLK11 propeptide includes only the P3–P1 positions); in these cases, additional residues of the corresponding KLK pre-sequence were included in the design (Fig. 1) so as to permit detectable mass changes upon analysis by SDS-PAGE (see below). In the case of the pro-KLK4 sequence, a native Cys residue at position P3 was substituted by Ser to avoid potential disulfide bond-mediated dimer formation. The insert and vector fragments were ligated and transformed into BL21(DE3) *Escherichia coli* for protein expression.

Expression of Pro-KLK Fusion Proteins—All expression and purification steps were performed at 4 °C following previously described procedures for the FGF mutant (42), with slight modification. Briefly, the cell lysate from 1-liter fermentation in minimal media was loaded directly onto a 2.5×15-cm column of nickel-nitrilotriacetic acid affinity resin (Qiagen, Valencia, CA). Elution was accomplished using a step gradient of 150 mM imidazole. Elution fractions were pooled and dialyzed against 50 mM sodium phosphate, 100 mM sodium chloride, 10 mM ammonium sulfate, and 0.5 mM EDTA, pH 7.5, using 6–8-kDa molecular weight cutoff dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA). The protein was then concentrated and loaded onto a Superdex 75 size-exclusion fast protein liquid chromatography column (Pharmacia) and eluted in the same buffer. The pooled protein was loaded onto a Sephadex G-25 superfine size-exclusion low pressure column for buffer exchange into 20 mM sodium phosphate, 0.15 M NaCl (PBS), pH 7.4. The purified protein was concentrated to 1.0 mg/ml, filtered through a 0.2-μm filter (Whatman Inc., Florham Park, NJ), snap-frozen in dry ice/ethanol, and stored at -80 °C prior to use.

have been identified as comprising the S3 to S1′ binding pockets (39) (corresponding to the binding of substrate P3 to P1′ positions). KLK1 is the most extensively characterized member of the KLK family, and ~80% of the binding energy of peptide substrates and inhibitors to KLK1 is contributed by interactions within the peptidase S2 to S1′ pockets (40). Furthermore, interactions within the S2 to S2′ pockets of KLK1 are primary determinants of its Met–Lys bond cleavage specificity, and interactions within the S1′ to S3′ pockets have been identified as important contributors to efficient hydrolysis of short peptide substrates (41). Together, these data identify interactions within the S3 to S3′ pockets as forming key structural determinants of general substrate specificity, binding, and hydrolytic efficiency for the KLK family. The design of the pro-KLK fusion protein includes the entire complement of "P" positions, in addition to the P1′ through P6′ residues, for each KLK (Fig. 1), thus spanning key determinants of propeptide recognition for hydrolysis.

Cloning of Pro-KLK Fusion Proteins—KLK propeptide fusion constructs made use of a previously reported mutant form of FGF combining the following mutations: L44F, M67I, L73V, A103G, Δ104–106, V109L, L111I, C117V, R119G, Δ120–122 (referred to as SYM6ΔΔ) (38). The properties of SYM6ΔΔ include a substantial (16.1 kJ/mol) increase in thermal stability; a 10-fold increase in solubility (>50 mg/ml), and the elimination of basic residues on two surface loops. This latter property, in conjunction with the increased stability, renders SYM6ΔΔ resistant to internal proteolysis by both trypsin- and chymotrypsin-like peptidases; references to "FGF" herein refer to this SYM6ΔΔ mutant form.

The pro-KLK peptide fusion construct utilizes residue positions 10–140 of FGF (numbering scheme of the 140 amino acid form of FGF) in addition to an amino-terminal His tag. Residue positions 1–9 of FGF are solvent-accessible and conformationally undefined in the x-ray structure (36, 37) (Fig. 2). NheI

Production of Mature KLK Proteins—All mature KLK proteins were derived from heterologous expression sources, and none were naturally isolated. Mature KLK1–3, -5–8, and -13 were expressed as recombinant proteins from an insect cell/baculovirus expression system as described previously (28, 43, 44). Briefly, the cDNA for the mature form of each of these KLK was inserted into the pBAC3 transfer vector (Novagen, Madison, WI) immediately 3' to a synthetic prepro-sequence containing a secretion signal, His tag, and enterokinase (EK; Asp$_4$-Lys) activation pro-sequence, respectively. Recombinant protein was produced from the TN5 (Invitrogen) insect cell line and purified using the His tag with nickel affinity resin. Cleavage of the propeptide EK recognition sequence was performed by EK digestion (Roche Diagnostics). Separation of EK and the propeptide from the mature KLK protein was accomplished using Sephadex G-50 gel filtration (GE Healthcare) in 40 mM sodium acetate, 100 mM NaCl, 0.01% Tween 20, pH 4.5. Proteins were concentrated to 1.0 mg/ml, snap-frozen in dry ice/ethanol, and stored at −80 °C prior to use. Sham purification experiments with nonexpressing insect cells demonstrated the absence of EK activity in the expected KLK elution fractions (data not shown). Molar extinction coefficients were calculated using the method of Gill and von Hippel (45). Mature KLK4, -11, -12, and -14 were obtained from R & D Systems (Minneapolis, MN) by activation of the pro-forms according to the manufacturer's instructions.

Production of Pro-KLK Proteins—Pro-KLK3, -4, -8, -11, -12, and -14 were obtained from R & D Systems. Recombinant pro-KLK1 protein was expressed from yeast (*Pichia pastoris*). Briefly, the gene for pro-KLK1 (GenBank™ accession number AY094609) was subcloned into the pPicZαA transfer vector (Invitrogen) and included a carboxyl-terminal His tag for purification purposes. The pPiczαA vector was subsequently introduced into yeast strain X-33, and a stable clone was selected following the manufacturer's instructions. Transformed yeast was grown in 10 g/liter yeast extract, 20 g/liter peptone, 100 mM potassium phosphate (pH 6.0), 13.4 g/liter yeast nitrogen base, 40 mg/liter biotin, and 5 ml/liter methanol in a 30 °C shaking incubator (250 rpm) for 2 days. Methanol induction (5 ml/liter) was maintained every 24 h. Media containing the secreted pro-KLK1 protein were harvested by centrifugation at 5,000 × *g*, followed by 0.2-μm filtration of the supernatant, and then loading directly onto nickel-nitrilotriacetic acid affinity resin (Qiagen Inc., Valencia, CA) (all purification steps were performed at 4 °C). Elution of the nickel-affinity resin was performed using a step gradient of 250 mM imidazole in 50 mM Tris-HCl, 150 mM NaCl, 0.01% Tween, pH 7.5. The eluted pro-KLK1 protein was pooled and dialyzed against 50 mM Tris, 150 mM NaCl, pH 8.0, using 6–8-kDa molecular weight cutoff dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA). The dialyzed protein was passed over a benzamidine-Sepharose affinity column (GE Healthcare) to remove any traces of contaminating yeast trypsin-like peptidases. The pro-KLK1 protein was subsequently loaded onto a Q-Sepharose anion-exchange column (GE Healthcare) and eluted with a 1 M NaCl gradient. The pooled fractions were concentrated to 1.0 mg/ml and were loaded onto a Superdex 75 size-exclusion column (GE Healthcare) and eluted with PBS. Purity and homogeneity of recombinant pro-KLK1 fractions were evaluated by 16.5% Tricine SDS-PAGE and amino-terminal sequencing. Pro-KLK1 fractions were subsequently pooled and concentrated to 1.0 mg/ml, snap-frozen, and stored at −80 °C. The molar extinction coefficient of the purified protein was calculated based on the spectroscopic method of Gill and von Hippel (45). To confirm the absence of contaminating host peptidase activity, purified pro-KLK1 was concentrated to 40 μM, incubated at 37 °C for 6 h at pH 8.0, and analyzed using 16.5% Tricine SDS-PAGE.

Pro-KLK6 was expressed as a recombinant protein from HEK293 human embryonic kidney epithelial cells as the expression host, as described previously (31). Briefly, pro-KLK6 was designed to be expressed as a fusion construct with a carboxyl-terminal Strep tag and His tag, respectively. The cDNA encoding human prepro-KLK6 was cloned into the pSecTag2/HygroB expression vector (Invitrogen). In this construct the native KLK6 secretion signal was utilized to direct secretion into the culture media. The HEK293 culture media were harvested 2 days after transfection, and the pro-KLK6 was purified by sequential affinity chromatography utilizing nickel-affinity resin and Strep-Tactin Superflow media (Qiagen, Valencia, CA), respectively. Purity and homogeneity of recombinant pro-KLK6 were evaluated by 16.5% Tricine SDS-PAGE and amino-terminal sequencing. Purified pro-KLK6 was dialyzed against 40 mM sodium acetate, 100 mM NaCl, 0.01% Tween 20, pH 4.5, concentrated to 1.0 mg/ml, snap-frozen, and stored at −80 °C. The extinction coefficient of pro-KLK6 was determined using the method of Gill and von Hippel (45). To confirm the absence of contaminating host peptidase activity, purified pro-KLK6 was concentrated to 40 μM, incubated at 37 °C for 6 h at pH 8.0, and analyzed using 16.5% Tricine SDS-PAGE.

Pro-KLK Fusion Protein Hydrolysis Assay—Pro-KLK fusion proteins and mature active KLK protein were diluted into PBS, pH 6.0 or 7.4, and combined in a 100:1 molar ratio, respectively, with a final pro-KLK fusion protein concentration of 40 μM. Samples were incubated at 37 °C for either 1 or 24 h, after which time they were immediately added to SDS sample buffer and boiled. The digestion samples (5.0 μg) were subsequently resolved using 16.5% Tricine SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. The stained gels were scanned, and the extent of hydrolysis was quantified against pro-KLK fusion protein standards using UN-SCAN-IT densitometry software (Silk Scientific, Orem, UT). The normalized percent hydrolysis is for the intact pro-KLK fusion protein; thus for samples with extensive fragmentation the percent hydrolysis is given in reference to the residual intact pro-KLK fusion protein.

The pro-KLK fusion proteins that exhibited proteolytic cleavage were subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis using a matrix of α-cyano-4-hydroxycinnamic acid and performed on an Axima CFR-plus mass spectrometer (Shimadzu Biotech, Columbia, MD). Those reactions exhibiting mass fragments inconsistent with the expected propeptide fragment were resolved on 16.5% Tricine SDS-PAGE, electroblotted onto polyvinylidene difluoride membrane, and subjected to amino-terminal peptide sequencing using an Applied Biosystems Pro-

cise model 492 protein sequencer (Applied Biosystems, Foster City, CA).

Autoactivation Assay of Pro-KLK3, -11, -12, and -14—All activation studies involving pro-KLK proteins were performed in duplicate. The assay for the autoactivation of pro-KLK3, -11, -12, and -14 essentially followed the procedure for the activation of these pro-forms by thermolysin (R & D Systems) with the substitution of the corresponding mature form of the KLK for thermolysin. Pro-KLK3 (4.0 μM) was incubated with 40 nM active KLK3 (100:1 molar ratio, respectively) in 50 mM Tris, 0.15 M NaCl, 0.05% Brij-35, pH 7.5, for both 1 and 24 h at 37 °C. The sample digest was subsequently diluted 1:10 into 50 mM Tris, 1 M NaCl, pH 8.0, and the mature KLK3 enzymatic activity generated was quantified by monitoring the rate of hydrolysis of 1 mM MeOSuc-Arg-Pro-Tyr-*p*-nitroanilide chromogenic substrate (MP Biomedicals, Aurora, OH) at 25 °C. Released *p*-nitroanilide chromogen was quantified by monitoring the increase in absorbance at 405 nM on a Varian Cary 200 UV-visible spectrophotometer, using an extinction coefficient of $E = 9{,}767$ M^{-1} cm^{-1} (46), and normalizing against a mature KLK3 standard curve. Controls included pro-KLK3 in the absence of added KLK3, KLK3 in the absence of added pro-KLK3, as well as buffer control.

Pro-KLK11 (4.0 μM) was incubated with 40 nM active KLK11 in 50 mM Tris, 10 mM CaCl$_2$, 0.15 M NaCl, pH 7.5, for 1 h at 37 °C. The generated mature KLK11 activity was subsequently measured using a thiobenzyl/DTNB-coupled cleavage assay. 0.1 mM each of D-Val-Leu-Lys-thiobenzyl substrate (MP Biomedicals, Aurora, OH) and DTNB (Ellman's reagent) chromogen were directly added to the reaction mixture. Hydrolysis of the Lys-thiobenzyl substrate by active KLK11 was quantified by monitoring generation of the DTNB thiolate ion at 405 nm with an extinction coefficient of $E = 13{,}600$ $M^{-1}cm^{-1}$ and a standard curve of mature KLK11. Controls included pro-KLK11 in the absence of added KLK11, KLK11 in the absence of added pro-KLK11, as well as buffer control.

Pro-KLK12 (4.0 μM) was incubated with 40 nM active KLK12 in 0.1 M Tris, 0.15 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35, pH 8.0, for 1 h at 37 °C. The sample digest was subsequently diluted 1:10 into 0.1 M Tris, 0.15 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35, pH 7.5 containing 0.1 mM Boc-Val-Pro-Arg-7-amino-4-methylcoumarin (Boc-Val-Pro-Arg-AMC; R & D Systems) substrate. The AMC fluorophore generated was quantified on a Varian Cary Eclipse fluorescence spectrophotometer using a standard curve of the Boc-Val-Pro-Arg-AMC substrate subjected to complete hydrolysis by overnight incubation with bovine trypsin. Normalization of KLK12 activity was performed using standards of KLK12 activated according to the manufacturer's protocol (R & D Systems). Controls included pro-KLK12 in the absence of added KLK12, KLK12 in the absence of added pro-KLK12, as well as buffer control.

Pro-KLK14 (4.0 μM) was incubated with 40 nM active KLK14 in 50 mM Tris, 10 mM CaCl$_2$, 0.15 M NaCl, 0.05% Brij-35, pH 7.5, for 1 h at 37 °C. The sample digest was subsequently diluted 1:10 into 50 mM Tris, 0.15 M NaCl, 0.05% Brij-35, pH 8.0, containing 0.1 mM Boc-Val-Pro-Arg-AMC substrate, and quantified as described above with normalization of KLK14 activity using KLK14 standards. Controls included pro-KLK14 in the absence of added KLK14, KLK14 in the absence of added pro-KLK14, as well as buffer control.

Activation Assays of Pro-KLK—Pro-KLK1 (4.0 μM) was incubated with 40 nM of KLK2 in 50 mM Tris, pH 7.4, for 1 h at 37 °C. The mature KLK1 activity produced in these reactions was assayed by the addition of 33.3 μM tosyl-Gly-Pro-Arg-AMC (GPR-AMC). Released fluorescence was monitored using an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA). The AMC fluorophore generated was quantified using a standard curve of the GPR-AMC subjected to complete hydrolysis by overnight incubation with bovine trypsin. Controls included assays of pro-KLK1 in the absence of added KLK2, KLK2, as well as buffer control.

Pro-KLK8 (4.0 μM) was incubated with 40 nM KLK12 in 50 mM Tris, pH 7.4, for 1 h at 37 °C. The sample digest was subsequently diluted 1:2 in 50 mM Tris, pH 9.0, containing a final concentration of 0.1 mM Boc-Val-Pro-Arg-AMC (VPR-AMC). The AMC fluorophore generated was quantified using a standard curve of the VPR-AMC substrate subjected to complete hydrolysis by overnight incubation with bovine trypsin. Fluorescence absorbance was monitored as described above for the GPR-AMC substrate. Controls included pro-KLK8 in the absence of added KLK12, KLK12, as well as buffer control.

Pro-KLK11 (4.0 μM) was incubated with 40 nM of either KLK3, -6, -12, or -14 in 50 mM Tris, 0.15 M NaCl, 0.05% Brij-35, pH 7.5 (TCNB buffer), for 1 h at 37 °C. The generated KLK11 enzyme activity was quantified using a coupled D-Val-Leu-Lys-thiobenzyl/DTNB assay as described above.

Pro-KLK12 (4.0 μM) was incubated with 40 nM of either KLK2, -5, -8, or -11 in 0.1 M Tris, 0.15 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35, pH 8.0, for 1 h at 37 °C. The sample digests were subsequently diluted 1:10 into 0.1 M Tris, 0.15 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35, pH 7.5, containing 0.1 mM Boc-Val-Pro-Arg-AMC substrate, and the generated KLK12 activity was quantified as described above.

Pro-KLK14 (4.0 μM) was incubated with 40 nM KLK11 in TCNB buffer for 1 h at 37 °C. The sample digest was subsequently diluted 1:10 into 50 mM Tris, 0.15 M NaCl, 0.05% Brij-35, pH 8.0, containing 0.1 mM Boc-Val-Pro-Arg-AMC. The KLK14 activity generated was assayed as described above for KLK12.

RESULTS

Purification of Pro-KLK Fusion Proteins—The pro-KLK fusion proteins were purified to apparent homogeneity, in each case, as determined by Coomassie Brilliant Blue-stained SDS-PAGE (see supplemental figures). Furthermore, the purified pro-KLK fusion proteins, in each case, showed no evidence of degradation because of the presence of contaminating *E. coli* peptidases after 24 h of incubation at 37 °C (at either pH 6.0 or 7.4) (see supplemental figures). Thus, the purified pro-KLK fusion proteins appeared suitable for use as substrates in proteolysis studies.

Proteolysis of Pro-KLK Fusion Proteins by Mature KLK—The 15 pro-KLK fusion proteins were subjected to hydrolysis by 12 different mature KLKs (100:1 molar ratio) at two different pH
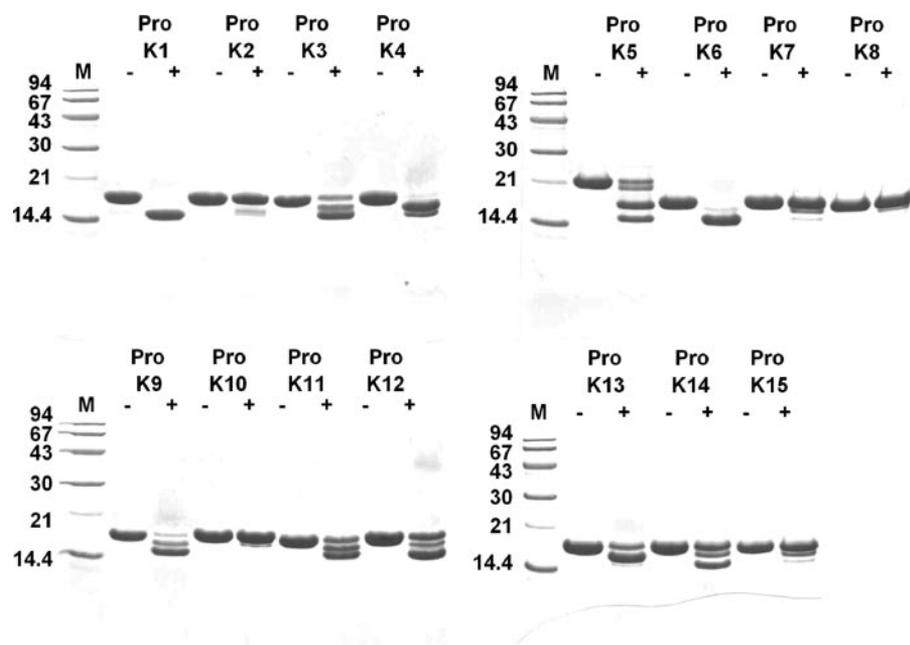
FIGURE 3. **Coomassie Brilliant Blue-stained 16.5% Tricine SDS-PAGE analysis of 100:1 molar ratio incubation of pro-KLK-(1–15) (abbreviated as "Pro-K") fusion proteins by mature KLK11 for 24 h at pH 7.4.** Note, at this concentration the KLK11 control does not yield a stainable band.

TABLE 1

Summary of mass spectrometry and N-terminal sequencing data for the 24-h KLK11 hydrolysis of the pro-KLK-(1–15) fusion proteins at pH 7.4 (see Fig. 3 and text)

Substrate	Experimental mass	Theoretical mass	N-terminal sequence
	Da	*Da*	
Pro-KLK1	1,880.18	1,878.89 (pro-peptide)	
Pro-KLK2		(No hydrolysis)	
Pro-KLK3	1,881.22	1,879.95 (pro-peptide)	
Pro-KLK4	14,809.43	14,811.55 (fusion protein)	
Pro-KLK5	4,197.81	4,196.78 (pro-peptide)	
Pro-KLK6	2,017.06	2,014.87 (pro-peptide)	
Pro-KLK7		(No hydrolysis)	
Pro-KLK8		(No hydrolysis)	
Pro-KLK9	2,025.27	1,952.85 (pro-peptide)	Ile-Gly-Ala-Glu-Glu P2', P3', P4', P5', P6'
Pro-KLK10		(No hydrolysis)	
Pro-KLK11	1,630.87	1,629.71 (pro-peptide)	
Pro-KLK12	1,814.10	1,812.83 (pro-peptide)	
Pro-KLK13	1,775.20	1,874.83 (pro-peptide)	Lys Val Leu Asn Thr P1, P1', P2', P3', P4'
Pro-KLK14	1,961.00	1,959.81 (pro-peptide)	
Pro-KLK15	1,826.91	1,743.74 (pro-peptide)	Leu Glu Gly Asp Glu P2', P3', P4', P5', P6'

agreement with the expected released propeptides (*i.e.* involving hydrolysis between the pro-KLK P1 and P1' positions). In the case of the hydrolysis of pro-KLK4 fusion protein by KLK11, a fragment representing the intact propeptide was not identified; however, a fragment corresponding to the correctly processed carboxyl-terminal fusion protein was identified, indicating that the propeptide could be released but that additional cleavages within the propeptide region may have degraded it. In the case of the pro-KLK9, -13, and -15 hydrolyses by KLK11, released propeptides of unexpected masses were identified, and the hydrolysis samples were therefore subjected to amino-terminal sequencing. The incubation of pro-KLK9 with KLK11 resulted in release of an aberrantly large propeptide because of hydrolysis between the P1' and P2' positions (*i.e.* hydrolysis after the P1' Ala residue; see Table 1 and Fig. 1). Similarly, the incubation of pro-KLK15 with KLK11 resulted in the release of an aberrantly large propeptide because of hydrolysis between the P1' and P2' positions (*i.e.* hydrolysis after the P1' Leu residue; see Table 1 and Fig. 1). In contrast, the incubation of pro-KLK13 fusion protein with KLK11 resulted in the release of an aberrantly small propeptide because of hydrolysis between the P2 and P1 positions (*i.e.* hydrolysis after the P2 Ser residue; see Table 1 and Fig. 1). In each of these cases, hydrolyses outside of the P1-P1' junction would not be expected to result in production of the active KLK. Hydrolysis within the P region of the propeptide would not result in activation but would not necessarily prevent subsequent activation through the action of a separate peptidase (with the ability to cleave at the P1–P1' junction). Hydrolysis within the "P-prime" region of the mature KLK would presumably result in proteolytic inactivation. Both types of proteolysis are therefore considered here simply to be "nonactivating."

The mass spectrometry and amino-terminal sequencing data for all reactions are provided as supplemental tables. For those hydrolyses constituting an "activating" event, as demonstrated by correct cleavage between P1 and P1' residues, scanning densitometry was performed to quantify the extent of hydrolysis. Tables 2 and 3 list the percent hydrolysis for the pro-KLK-(1–15) fusion proteins by the set of mature KLK utilized in the present study (at both pH 6.0 and 7.4, and for both 1- and 24-h incubations).

Activation Studies of Pro-KLK—In describing the results of the present study, if KLK "*X*" is capable of activating pro-KLK *X*, this is referred to as "autoactivation"; if KLK *X* is capable of activating pro-KLK *Y*, this is referred to as "cross-activation" of *Y* by *X*. The pro-KLK activation assays are coupled assays that involve an initial activation step, followed by a second brief

values (7.4 and 6.0), and for two different incubation periods (1 and 24 h), resulting in the analysis of 1,440 samples (including controls). Scanned images of the SDS-polyacrylamide gels (133 total) are provided as supplemental figures; however, Fig. 3, involving incubation of the pro-KLK-(1–15) fusion proteins with mature KLK11, at pH 7.4 for 24 h, is included here as an illustrative example.

Incubation of the pro-KLK-(1–15) fusion proteins with mature KLK11 resulted in significant hydrolysis of the pro-KLK1, -3–5, -6, -9, and -11–15 fusion proteins, with little or no hydrolysis observed for the pro-KLK2, -7, -8, and -10 fusion proteins (Fig. 3). Those pro-KLK fusion proteins exhibiting hydrolysis by KLK11 were subjected to mass spectrometry analysis (Table 1). Hydrolysis of pro-KLK1, -3, -5, -6, -11, -12, and -14 fusion proteins yielded mass fragments in excellent

TABLE 2

Percent hydrolysis of pro-KLK-(1–15) fusion proteins for 100:1 molar ratio incubation with mature KLKs (1000:1 with KLK7) at pH 6.0/7.4 for 1 h at 37 °C

	P1	KLK1	KLK2	KLK3	KLK4	KLK5	KLK6	KLK7	KLK8	KLK11	KLK12	KLK13	KLK14
Residue 189		Asp	Asp	Ser	Asp	Asp	Asp	Asn	Asp	Asp	Asp	Asp	Asp
Pro-KLK1	Arg		69/95	0/5	96/96	92/99	43/44	0/2	5/6	7/55	87/95	10/18	90/92
Pro-KLK2	Arg		99/99	2/6	100/99	100/100	53/50	6/9	3/4		100/100	31/48	100/100
Pro-KLK3	Arg		81/94	0/5	94/86	87/100	15/98	24/82	0/6	2/21	79/100	19/62	98/100
Pro-KLK4	Gln									0/4			
Pro-KLK5	Arg		43/37		26/43	54/82	6/7		1/5	0/4	11/21		86/98
Pro-KLK6	Lys				55/61	17/45				35/37			18/23
Pro-KLK7	Lys					8/26					0/4		
Pro-KLK8	Lys					10/16					2/6		
Pro-KLK9	Arg	3/2	91/64	21/27	98/98	92/100	99/46				76/98	6/11	93/100
Pro-KLK10	Arg						2/0						5/21
Pro-KLK11	Arg		50/60	4/16	97/92	94/100	36/44		9/29	6/7	63/99	6/20	100/100
Pro-KLK12	Lys		3/6		98/95	16/100			0/3	0/7	65/92		64/97
Pro-KLK13	Lys				17/16	2/12					29/55		6/16
Pro-KLK14	Lys				46/43	5/18				0/5	1/5		1/4
Pro-KLK15	Lys				12/11	6/38					12/31		5/1

TABLE 3

Percent hydrolysis of pro-KLK(1–15) fusion proteins for 100:1 molar ratio incubation with mature KLKs (1000:1 with KLK7) at pH 6.0/7.4 for 24 h at 37 °C

	P1	KLK1	KLK2	KLK3	KLK4	KLK5	KLK6	KLK7	KLK8	KLK11	KLK12	KLK13	KLK14
Residue 189		Asp	Asp	Ser	Asp	Asp	Asp	Asn	Asp	Asp	Asp	Asp	Asp
Pro-KLK1	Arg		95/100	23/63	99/100	98/100	98/93	0/3	34/60	65/99	98/96	57/70	99/92
Pro-KLK2	Arg		100/100	20/60	100/100	100/100	100/100	37/54	33/53		99/100	98/100	100/100
Pro-KLK3	Arg		99/100	18/75	100/100	100/100	98/98	99/100	37/71	12/54	100/100	99/98	100/100
Pro-KLK4	Gln			6/17						0/23			
Pro-KLK5	Arg		97/85	11/14	99/99	100/99	70/28		26/31	0/45	48/88	6/5	100/100
Pro-KLK6	Lys		11/0		100/99	86/77	25/0		4/0	44/99	3/11	5/0	99/100
Pro-KLK7	Lys					67/45	9/0				12/20		
Pro-KLK8	Lys					67/95					22/30		
Pro-KLK9	Arg	32/19	99/98	84/95	99/99	100/100	100/95	12/10	22/25		94/99	43/51	100/100
Pro-KLK10	Arg			2/0		11/0	37/0				7/14		54/86
Pro-KLK11	Arg		99/99	56/58	100/100	99/100	98/99		82/87	22/37	100/100	70/81	100/100
Pro-KLK12	Lys		50/52		100/100	89/100	10/0		8/24	30/58	100/99	2/9	100/100
Pro-KLK13	Lys		10/0		69/99	36/36					93/100		41/89
Pro-KLK14	Lys				100/100	59/40				34/50	15/11		23/55
Pro-KLK15	Lys				49/61	62/74					56/82		25/80

detection step using chromogenic or fluorogenic substrates. The activation was performed in PBS, pH 7.4, for 1 h at 37 °C, or in related buffers (*e.g.* Tris, pH 7.5–8.0, with specific salt or detergent additives) if additional information was available (*e.g.* from a manufacturer) indicating the particular KLK in question would be more stable in the presence of such additives. The conditions for the comparatively rapid secondary detection step varied depending on the substrate being used (see "Experimental Procedures").

Autoactivation of Pro-KLK—The hydrolysis data for the pro-KLK fusion proteins by mature KLK (Tables 2 and 3) indicate the potential for efficient autoactivation (*i.e.* 82–99% hydrolysis) for KLK2, KLK5, and KLK12. Autoactivation for these three KLK has been reported previously in the literature (15, 21, 47–50). The results also indicate the potential for autoactivation , albeit at a comparatively reduced rate of efficiency, for KLK3, -11, and -14 (Tables 2 and 3). The ability of KLK3, -11, and -14 to autoactivate was evaluated by combining the pro- and mature forms of these KLKs and assaying for the generation of mature KLK activity. No evidence was found for autoactivation of KLK3 under the conditions tested (see "Experimental Procedures"). Incubation of pro-KLK11 with mature KLK11 resulted in the generation of ~220 ± 30 nM mature KLK11 activity, significantly above that of the control samples. The incubation of

pro-KLK14 with mature KLK14 resulted in the production of 30 ± 9 nM KLK14 (Fig. 4).

Cross-activation Studies of KLK—A subset of possible KLK cross-activation relationships identified using the pro-KLK fusion protein hydrolyses was evaluated utilizing available pro- and mature forms of the KLK. These studies included evaluation of the activation of pro-KLK1 by KLK2 (Fig. 5, *panel A*), pro-KLK3 by KLK11 (Fig. 5, *panel B*), pro-KLK8 by KLK12 (Fig. 5, *panel C*), pro-KLK11 by KLK3, -6, -12, and -14 (Fig. 6, *panel A*), pro-KLK12 by KLK2, -5, -8, and -11 (Fig. 6, *panel B*), and pro-KLK14 by KLK11 (Fig. 5, *panel D*). The results of these incubations are summarized along with the autoactivation studies in Fig. 7.

DISCUSSION

By using pro-KLK fusion proteins, we characterized the activation profile of the 15 pro-KLKs using a total of 12 mature KLKs. These hydrolytic results suggested 15 novel KLK activation relationships; of these, 11 demonstrated activation *in vitro* using native pro-KLK proteins. To our knowledge, these activation relationships are previously unreported and therefore considerably expand our understanding of the potential for KLK activation relationships. This in turn permits vastly more extensive KLK activation cascades to be proposed.
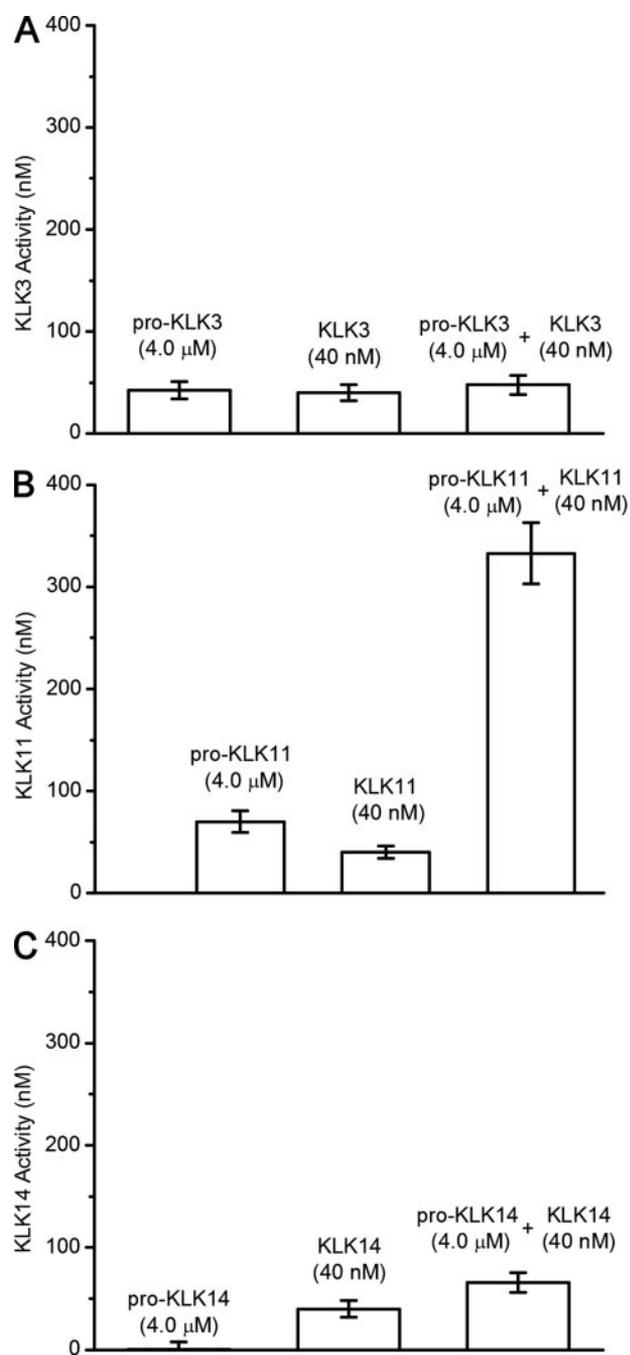
FIGURE 4. **Evaluation of the autolytic activation potential for KLK3 (*panel A*), KLK11 (*panel B*), and KLK14 (*panel C*).** 4.0 μM pro-KLK, in each case, was incubated with 40 nM mature KLK (100:1 molar ratio) for 1 h, pH 7.4, and at 37 °C.

glycosylation in comparison with the natural forms. In the case of recombinant KLK1, the site of glycosylation is known to be heterogeneous, but the kinetic properties are indistinguishable from those of naturally derived KLK1 (44). However, very few naturally isolated human KLKs have been characterized, and it remains to be seen if the catalytic properties of recombinant KLK are substantially different from those of their natural counterparts, particularly as regards the heterogeneous glycosylation.

The propeptides exhibiting the least hydrolysis by the KLKs tested were pro-KLK4, -8, and -10. The pro-sequence of KLK4 proved to be the most difficult to hydrolyze. Pro-KLK4 contains an atypical Gln at the P1 position, and limited hydrolysis of the pro-KLK4 fusion protein was observed only after 24 h of incubation with KLK3 or KLK11. Given the relatively poor hydrolysis of pro-KLK4 by the KLK tested, a separate peptidase may function *in vivo* to activate pro-KLK4. However, the pro-KLK4 sequence was also the one where the native P4 residue (Cys) was altered (to Ser) to avoid thiol-mediated dimer formation. This substitution, although conservative, may nonetheless influence recognition and hydrolysis by an activating peptidase. Pro-KLK8 and -10 also proved resistant to hydrolysis (Fig. 7). Pro-KLK8 contains a Lys residue at the P1 position, along with pro-KLK6–7 and -12–15 (Fig. 1). The pro-KLK peptides with Lys at the P1 position exhibit a more selective hydrolytic profile, in comparison with those with Arg. Pro-KLK8 also contains acidic residues at both the P2 and P3 positions, and these may confer added selectivity for the activating protease. The resistance of pro-KLK10 to hydrolysis appears unexpected in that it contains an Arg in the P1 position (Fig. 1). However, pro-KLK10 is atypical in that it contains an acidic Asp residue at the P2′ position; all other pro-KLK sequences contain a hydrophobic P2′ residue. From the crystal structure of human KLK1 it is known that the S2′ pocket is a hydrophobic cleft formed by residues Phe-40, Phe-151, and Gly-193 (44). The hydrophobic nature of these residues is highly conserved in the KLK family (3) and would therefore be expected to confer a preference for substrate hydrophobic P2′ residues. Thus, the acidic P2′ Asp residue in pro-KLK10 may confer selectivity for a novel activating protease.

KLK1, also referred to as "true kallikrein," is able to cleave various prohormones and bioactive peptides, including kininogen, proinsulin, prorenin, and procollagenase (51), and plays a major role in inflammation and heart disease. Lysyl-bradykinin, or kallidin, is a decapeptide produced by the proteolytic action of KLK1 from low molecular weight kininogen via cleavage between two specific bonds involving Met-Lys and Arg-Ser sequences, and this dual specificity of KLK1 is a characteristic property of this KLK (23). With regard to general properties of a particular KLK as assessed by the pro-KLK fusion hydrolysis results, the data in Tables 2 and 3 suggest that KLK1 plays little, if any, role in broader activation cascades involving other members of the KLK family.

The hydrolysis results in Tables 2 and 3 show that KLK4, -5, -11, -12, and -14 exhibit generally broad specificity toward Arg *versus* Lys residues in the substrate P1 position (with KLK5 potentially exhibiting the broadest specificity). These members of the KLK family may therefore function as "universal activa-
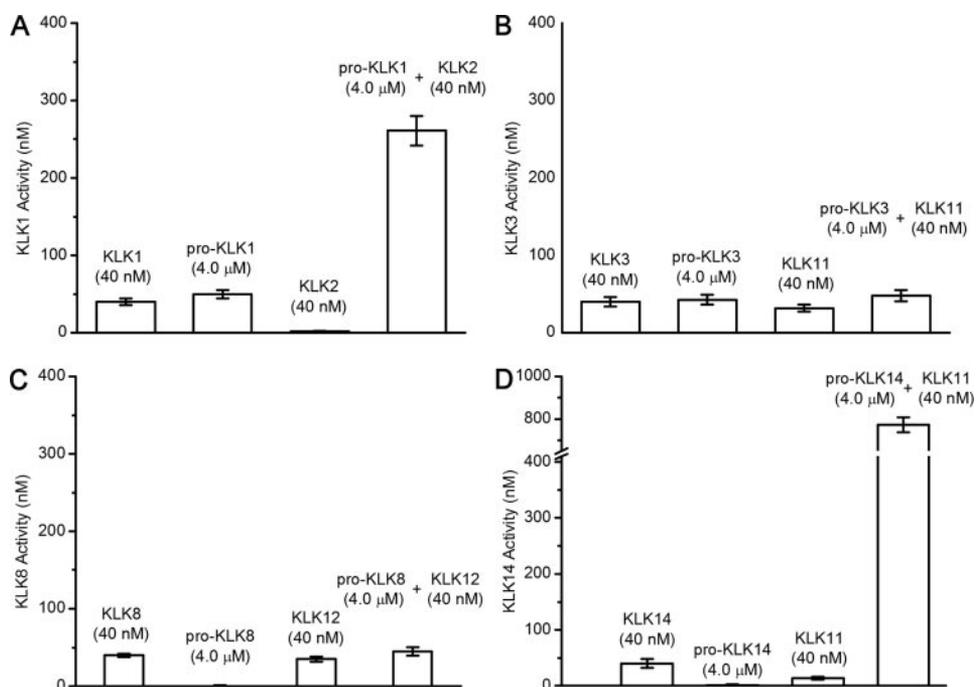
The data generated in this study permit a comparative evaluation of the ability of the different KLKs to correctly process a particular KLK propeptide, and conversely, the data also permit a comparative evaluation of the ability of a given KLK to process all 15 KLK propeptides. The ability of the different KLKs to correctly process a particular KLK propeptide can be readily discerned from the data within *each row* in Fig. 7; similarly, the ability of a given KLK to process all 15 KLK propeptides can be understood from the data in *each column* in Fig. 7. We note that all the KLK proteins utilized in the present study were recombinantly derived and likely contain atypical and heterogeneous

FIGURE 5. **Evaluation of activation of pro-KLK1 by KLK2 (*panel A*), pro-KLK3 by KLK11 (*panel B*), pro-KLK8 by KLK12 (*panel C*), and pro-KLK14 by KLK11 (*panel D*).** 4.0 μM pro-KLK, in each case, was incubated with 40 nM (100:1 molar ratio) mature KLK11 for 1 h, pH 7.4, and at 37 °C.

FIGURE 6. **Evaluation of activation of pro-KLK11 by KLK3, -6, -12, and -14 (*panel A*) and activation of pro-KLK12 by KLK2, -5, -8, and -11 (*panel B*).** 4.0 μM pro-KLK, in each case, was incubated with 40 nM (100:1 molar ratio) mature KLK for 1 h, pH 7.4, and at 37 °C. *, pro-KLK12 has autolytic activity, and a background control incubation was subtracted from all samples.

Lys basic residues but also after the Gln P1 residue of KLK4, as well as unexpected (*i.e.* non-P1 site) hydrolyses after Ala (pro-KLK9), Ser (pro-KLK13), and Leu (pro-KLK15) residues. These results show that KLK11 is capable of dual substrate specificity (both trypsin-like and chymotrypsin-like) and is in good agreement with the P1 specificity of KLK11 as determined using a positional scanning combinatorial library of tetrapeptides (52). The dual substrate specificity of KLK11 is similar to KLK1, but KLK11 exhibits a much broader specificity in contrast to KLK1.

KLK2, -6, -7, -8, and -13 exhibit primarily Arg-specific hydrolyses. This set of KLKs therefore is unlikely to be able to activate those pro-KLKs with Lys in the P1 position. Because among this set, KLK6 – 8 and -13 contain a Lys at the P1 position of their pro-sequence, this result suggests that these KLKs are unlikely to efficiently self-activate, and require a distinct (Lys-specific) protease for activation.

Several hydrolyses were observed to result in pro-KLK peptide cleavage amino-terminal to the P1–P1′ junction, including the hydrolysis of pro-KLK4 by KLK4, pro-KLK5 by KLK4, pro-KLK7 by KLK4 and KLK14, pro-KLK8 by KLK4 and KLK14, and pro-KLK13 by KLK11 (supplemental Tables 1–15). Although such hydrolyses are not expected to produce active KLK, the modification of the propeptide sequence might modulate the efficiency of subsequent hydrolysis by the activating protease. KLK4 appears to be most active regarding modification of the pro-sequence of other KLKs. We note that KLK5 has the longest pro-sequence and can be proteolytically modified by KLK4. KLK5 is reported to be capable of autolytic activation (15, 21); however, potential effects upon the efficiency of such self-acti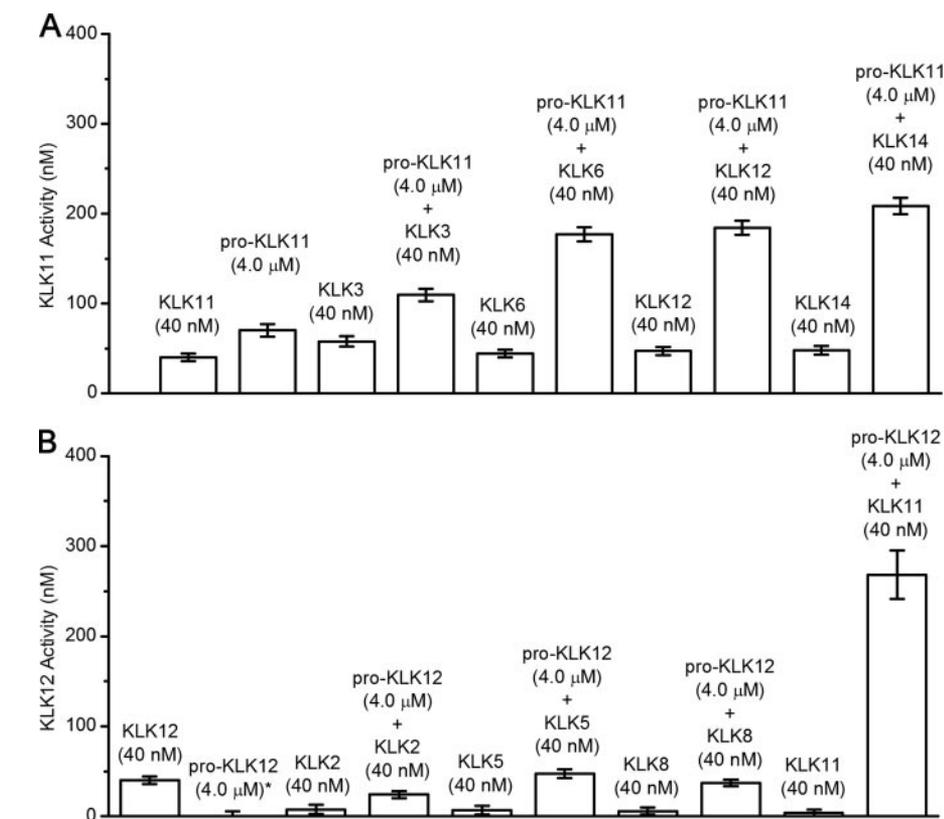vation after intermediate processing of the propeptide by KLK4 are unknown. Related to the unique length of the KLK5 pro-sequence, we note that the pro-sequence may adopt a unique conformation in association with the mature

tors" of members of the KLK family, in that they appear capable of hydrolyzing almost all of the pro-KLK sequences. We note that KLK11 exhibited ability to hydrolyze not only after Arg and

FIGURE 7. **Summary of pro-KLK fusion protein digest results (pH 7.4 for 1 h at 37 °C) represented in *gray scale* (*white* = 0%, *black* = 100% hydrolysis).** The *cross-hatches* indicate those activation relationships reported in the literature for pro-KLK (15, 19, 21, 27, 31, 48, 49, 50), and the *single hatches* indicate those activation relationships for which evidence is provided in this report for hydrolysis of pro-KLK.

panel A). Because these KLK are co-expressed in prostate, breast, and salivary gland (3), we postulate that this is a functionally relevant regulatory interaction. The ability of KLK6, -12, and -14 to activate pro-KLK11 (Fig. 6, *panel A*) also represents novel cross-activation relationships and has relevance to the physiology of prostate, uterus, thymus, thyroid, salivary glands, CNS, trachea, lung, and breast (all tissues co-expressing KLK11 with one or more of these KLKs (3)). The ability of KLK11 to activate pro-KLK14 (Fig. 5, *panel D*) is yet another novel cross-activation relationship, relevant to the physiology of uterus, thymus, thyroid, CNS, and breast (3). Similarly, the ability of KLK2, -5, -8, and -11 to activate pro-KLK12 is relevant to a variety of tissues co-expressing pro-KLK12 with one or more of these KLKs (including prostate, lung, breast, testis, and CNS

KLK5 protein that influences its hydrolysis. If so, this unique aspect of the KLK5 pro-sequence would not be reproduced in the pro-KLK fusion construct.

Several hydrolyses of the pro-KLK sequences were observed to occur in the P prime region carboxyl-terminal to the P1–P1′ junction. These included the hydrolysis of pro-KLK9 by KLK11, pro-KLK10 by KLK4, pro-KLK13 by KLK4, pro-KLK15 by KLK4, and after 24 h of digest at pH 6.0 pro-KLK12 by KLK7, pro-KLK13 by KLK3, and pro-KLK15 by KLK11 (supplemental Tables 1–16). These hydrolyses likely result in nonactivable (*i.e.* permanently inactive) forms of the KLK. Autolysis resulting in functional inactivation has been reported for KLK6 (28), and the above hydrolyses represent additional potential regulatory inactivation events.

Consideration of the general specificities exhibited by the KLK identifies a sub-category that may be capable of autoactivation and those that likely require cross-activation by another peptidase. The hydrolytic profiles presented in Tables 2 and 3 suggest that KLK2, -5, and -12 should be capable of efficient autoactivation, and this property has been reported for each of these KLKs (15, 21, 47–50). Novel autoactivation , albeit at a lower relative efficiency, is suggested by the results for KLK3, -11, and -14 (Tables 2 and 3). Evidence of autoactivation was subsequently observed for KLK11, and to a lesser extent KLK14, but not for KLK3 (Fig. 4). Thus, the propeptide of KLK3 may interact with the KLK3 protein to restrict hydrolysis.

The data in Tables 2 and 3 indicate the potential for specific cross-activation relationships between the KLK and, of particular interest, involving the action of the broad specificity KLKs (*i.e.* universal activators) in activating the Lys-containing pro-KLK. Several of these relationships were evaluated. The ability of KLK2 (an Arg-specific KLK) to activate pro-KLK1 (having a P1 Arg) is another novel cross-activation relationship (Fig. 5,

(3)). Cross-activation demonstrated in the pro-KLK fusion protein, but not observed for actual pro-KLK proteins, under the conditions tested include the activation of pro-KLK3 by KLK11, pro-KLK8 by KLK12, pro-KLK11 by KLK3, and proKLK12 by KLK8.

One potential consequence of the fusion construct approach that might influence catalysis is the lack of interactions between the propeptide and native peptidase domain. For example, full recognition between an activating peptidase and pro-KLK may involve participation of additional "exo-sites" (53) within the mature KLK. Additionally, interactions between the propeptide and KLK peptidase domain may affect the accessibility or electrostatic properties of the propeptide, thus influencing binding and hydrolysis by an activating peptidase. Therefore, the efficiency of hydrolysis observed for the propeptide fusion proteins is likely to be modulated (either increased or decreased) in comparison with the native pro-KLKs. Four of the activation reactions involving native pro-KLK proteins proceeded at rates similar to those observed for the pro-KLK fusion proteins, six were slower, and one (the activation of pro-KLK14 by KLK11) was significantly faster (Table 4). Therefore, overall the pro-KLK fusion proteins provided relevant lead information in the majority of cases, but the actual rates of hydrolysis were modulated when using the native pro-KLK protein as substrate. The turnover numbers provide in Table 4 are derived, in each case, for the pro-KLK (substrate) concentration of 4.0×10^{-6} M. Further study is required to understand the relationship between this substrate concentration and K_m, as well as the full determination of the Michaelis-Menten constants for these enzyme/substrate pairs.

Although the turnover numbers in Table 4 may be considered slow in comparison with other proteolytic systems, the temporal nature of regulatory cascades involving the KLK

TABLE 4

Activation of pro-KLKs (4.0 μM) by mature KLKs (40 nM in PBS, pH 7.4, for 1 h at 37 °C (normalized to account for any basal level hydrolysis of controls)

Pro-KLK	Mature-KLK	Pro-KLK activated	Hydrolysis	Reaction rate
		nM	$\%^a$	min^{-1}
Pro-KLK1	KLK2	210 ± 19	5.5 (99)	8.75×10^{-2}
Pro-KLK3	KLK3	0	0 (5)	0
Pro-KLK3	KLK11	0	0 (21)	0
Pro-KLK8	KLK12	0	0 (6)	0
Pro-KLK11	KLK3	0	0 (16)	0
Pro-KLK11	KLK6	70 ± 8	1.8 (44)	2.92×10^{-2}
Pro-KLK11	KLK11	220 ± 30	5.5 (7)	9.17×10^{-2}
Pro-KLK11	KLK12	75 ± 8	1.9 (99)	3.13×10^{-2}
Pro-KLK11	KLK14	90 ± 9	2.3 (100)	3.75×10^{-2}
Pro-KLK12	KLK2	16 ± 4	0.4 (6)	6.67×10^{-3}
Pro-KLK12	KLK5	40 ± 5	1.0 (100)	1.67×10^{-2}
Pro-KLK12	KLK8	31 ± 8	0.8 (3)	1.29×10^{-2}
Pro-KLK12	KLK11	264 ± 27	6.6 (7)	1.10×10^{-1}
Pro-KLK14	KLK11	720 ± 36	18.0 (5)	3.00×10^{-1}
Pro-KLK14	KLK14	30 ± 9	0.8 (4)	1.25×10^{-2}

^{a} Values in parentheses are for hydrolyses of related pro-KLK fusion proteins under equivalent incubation conditions.

might be significantly different compared with more familiar proteolytic cascades (*e.g.* thrombostasis). In thrombostasis the relevant signal generated by the proteolytic cascade manifests over a time frame of seconds and is essential for the function of that particular cascade. However, functionally relevant regulatory cascades involving the KLK may extend over days or weeks. An activation cascade has been proposed in human skin (stratum corneum) and involving KLK5, -7, and -14 in the regulation of desquamation (15). This process involves effective activation rates that occur on the time scale of hours to days, regulated by pH changes in the skin, and is quite slow in comparison with the more familiar proteolytic cascades. KLK6 has been reported to be involved in the process of skin carcinogenesis via the alteration of ECM proteins to support tumor cell growth and invasion (54). In this case, KLK6 appears functionally up-regulated throughout the entire pre-cancerous and invasive cancerous stages (*i.e.* spanning a period of days and weeks). Furthermore, there is evidence to suggest that KLK6 activity associated with spinal cord trauma, including dynamic modification of the ECM affecting the capacity for axon outgrowth, occurs over a temporal period of days (55). Indeed, one of the most important potential uses of the KLK is diagnostic and prognostic cancer biomarkers (8), suggesting functionally relevant activities that are likely to be temporally extensive. It appears unlikely, therefore, that regulatory cascades involving the KLK resolve over a short time frame, in this case, comparatively slow reaction rates are potentially physiologically relevant.

A phenomenon known as local acidosis occurs at sites of tissue damage or inflammation where the local pH falls to pH <6. This phenomenon has been reported to occur at sites of inflammation, cardiac ischemia, infarcted brain, and in the brains of Alzheimer patients (56–59). To evaluate the potential for altered hydrolysis profiles in response to physiological conditions of inflammation or disease, hydrolysis of the pro-KLK fusion proteins at pH 6.0 was also investigated (Tables 2 and 3). In general, lowering the pH decreases hydrolytic activity, but primarily for KLK3, -5, -8, -11, -12, and -14. This likely reflects a general pH optimum for these KLKs that is in the basic regime. In contrast, the hydrolytic activity of KLK1, -2, -7, and

-13 appear to be generally pH-independent. Notably, the rate of hydrolysis of several pro-KLKs by KLK6 generally increases with acidic conditions; thus, KLK6 activity may be stimulated under physiological conditions of inflammation, hypoxia, and tissue damage. Notably, KLK6 activity has been associated with CNS ischemia (60), trauma (55), and inflammatory demyelination in the CNS (17, 61). The hydrolysis of pro-KLK5 and -10 exhibits the greatest increase in response to acidic conditions (Tables 2 and 3), and because these KLKs are co-expressed with KLK6 in the CNS, we postulate that this response is of physiological relevance. The buffer conditions utilized in this study are unlikely to be optimal in each case, as studies have shown that salts and pH can have a substantial influence upon individual KLK activity and indeed may serve to regulate their activity (15, 62).

The potential for reciprocal cross-activation between KLK, leading to geometric signal amplification, is of great interest to elucidate. From analysis of the pro-KLK fusion hydrolyses significant reciprocal cross-activation is predicted for KLK pairs 2, 5; 2, 12; 3, 11; 5, 6; 5, 8; 5, 11; 5, 12; 5, 14; 6, 11; 11, 12; and 11, 14 (see Tables 2 and 3). However, we were unable to detect activation of pro-KLK3 by KLK11, and so we have no evidence for this pair of KLKs to participate in reciprocal cross-activation. KLK5 and KLK14 are co-expressed in the stratum corneum of skin and the ability of KLK5 to activate pro-KLK14, and similarly for KLK14 to activate pro-KLK5, has been reported (15) (although the ability of KLK5 to activate pro-KLK14 has been called into question (63)). We demonstrate in this report the ability of KLK11 to activate pro-KLK14 and the ability of KLK14 to activate pro-KLK11. KLK11 and KLK14 are co-expressed in uterus, thymus, thyroid, breast, and CNS (both being strongly expressed in CNS) (3). Thus, we postulate that reciprocal cross-activation is of physiological relevance for this pair of KLKs in these tissues (particularly CNS). We also demonstrate the ability of KLK11 to activate pro-KLK12 and the ability of KLK12 to activate pro-KLK11. KLK11 and KLK12 are co-expressed in salivary glands, thyroid, thymus, uterus, prostate, breast, lung, trachea, and CNS (with both being strongly expressed in salivary glands, thymus, uterus, prostate, and lung) (3). Thus, we postulate that reciprocal cross-activation is of physiological relevance for this pair of KLKs in these tissues. Also in this study we demonstrate the ability of KLK6 to activate pro-KLK11. Although we do not yet have evidence for the reciprocal cross-activation of pro-KLK6 by KLK11, it remains a possibility.

Knowledge of the pattern of co-expression of the KLK in different tissues, in combination with the potential activation relationships identified in Tables 2 and 3 allows for tissue-specific activation interactions ("KLK regulatory cascades") to be postulated. Several members of the KLK family are known to be expressed in the CNS. Immunoreactive KLK1 has been reported to be present in developing rat brains (64). KLK5 cDNA has been identified in brain, placenta, and kidney (65). KLK6 was identified from screening of cDNA from a human Alzheimer-diseased brain (66), as well as originally identified in breast, and later shown to be present in brain (67, 68) and also identified from screening of rat spinal cord cDNA (69). Western blotting studies have shown that KLK6 is present in the inactive pro-form in cerebrospinal fluid (70). KLK8 was initially
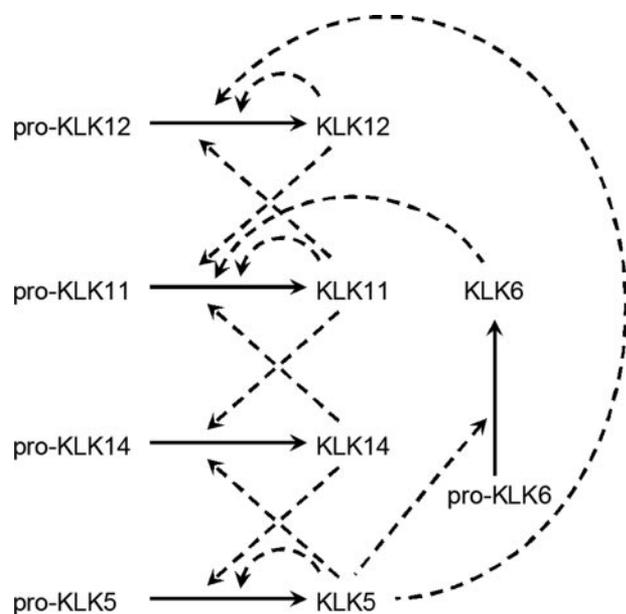
FIGURE 8. **A compilation of known activation interactions between KLK5, -6, -11, -12, and -14, previously reported or demonstrated here.** These KLK are expressed within the CNS and therefore potentially form a tissue-specific activation cascade.

identified from screening of cDNA from the limbic system of mouse brain (71). KLK11 (also known as hippostasin) cDNA has been characterized from both prostate and brain (72). KLK12 mRNA is found in a wide variety of tissues, including the brain (73). The tissue distribution levels of KLK14 mRNA are highest within the CNS (74). RT-PCR of brain and spinal cord shows that KLK1, -2, -5–10, and -12–14 are expressed in CNS (7). A review of the reported expression levels of the different KLK in human tissues compiled by Diamandis and co-workers (3) lists KLK5–9, -11, and -14 as exhibiting the greatest level of expression in the CNS. Thus, a significant subset of KLK is expressed in the CNS and therefore has the potential to participate in a regulatory cascade.

It can be appreciated from the data in Tables 2 and 3 that there exists the potential for complex activation cascades involving members of the KLK family, including autoactivation, cross-activation, and reciprocal cross-activation relationships. Using only data previously reported, or described herein, for confirmed activation interactions between mature and native pro-KLK proteins, we have constructed one such possible activation cascade relevant to the CNS (Fig. 8). The activation of any of the KLK in this diagram would potentially result in amplification of the other members. However, we note that KLK5 has the broadest activation profile, and exhibits one of the highest expression levels in CNS (7); thus, this KLK may play a central role in initial stimulation of a KLK activation cascade in this tissue. KLK14 is reportedly 1–2 orders of magnitude more efficient than KLK5; however, KLK14 appears able to only weakly self-activate (Fig. 4, *panel C*) and would therefore likely be dependent upon KLK5 (15) (or KLK11) for efficient activation.

In addition to activation, the functional regulation of KLK activity would naturally include the interaction of specific inhibitors, along with proteolytic inactivation via specific hydrolyses at internal sites within the active KLK. Notably, both KLK6 and -11 have been reported to autolytically inactivate subsequent to activation (28, 29). Thus, there are known mechanisms by which the proposed cascade would be self-regulated, even in the absence of specific inhibitors, following an initial stimulation event. The initial activation of pro-KLK5 in the CNS is a matter of speculation, but it may involve KLK14 or the action of a distinct peptidase. The proposed activation cascade also suggests that effective inhibition of specific KLK might be achieved by inhibition of the "upstream" activating peptidase. Inhibition of KLK6 has been shown to ameliorate the process of inflammatory demyelination in animal models (17); thus, inhibition of KLK5 may also serve to effectively inhibit the activity of KLK6 via inhibition of pro-KLK6 activation.

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