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Human kallikrein 6 activity is regulated via an autoproteolytic mechanism of activation/inactivation

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Summary

Human kallikrein 6 (protease M/zyme/neurosin) is a serine protease that has been suggested to be a serum biomarker for ovarian cancer and may also be involved in pathologies of the CNS. The precursor form of human kallikrein 6 (pro-hK6) was overexpressed in *Pichia pastoris* and found to be autoprocessed to an active but unstable mature enzyme that subsequently yielded the inactive, self-cleavage product, hK6 (D^{81}-K^{244}). Site-directed mutagenesis was used to investigate the basis for the intrinsic catalytic activity and the activation mechanism of pro-hK6. A single substitution $R^{80} \rightarrow Q$ stabilized the activity of the mature enzyme, while substitution of the active site serine ($S^{197} \rightarrow A$) resulted in complete loss of hK6 proteolytic activity and facilitated protein production. Our data suggest that the enzymatic activity of hK6 is regulated by an autoactivation/autoinactivation mechanism. Mature hK6 displayed a trypsin-like activity against synthetic substrates and human plasminogen was identified as a putative physiological substrate for hK6, as specific cleavage at the plasminogen internal bond S^{460}-V^{461} resulted in the generation of angiostatin, an endogenous inhibitor of angiogenesis and metastatic growth.

Keywords: angiostatin; autoactivation; human kallikrein 6; serine protease; site-directed mutagenesis.

Introduction

Human kallikrein 6 (hK6) was originally identified by differential display as being overexpressed in a primary breast tumor but inactivated in the metastatic tumor of the same patient and was named protease M (Anisowicz et al., 1996). In addition, it was found to be overexpressed in primary ovarian tumor tissues and cell lines but down-regulated in corresponding cells derived from tumor metastases. Based on this expression pattern, it was suggested that protease M may play a role in cancer metastasis (Anisowicz et al., 1996). The gene encoding protease M (KLK6) was localized by somatic cell hybrid and FISH analyses to human chromosome 19q13.3 (Anisowicz et al., 1996; Yousef et al., 1999), where it co-localizes with all other human kallikrein and kallikrein-like genes. The same gene was also cloned from an adenocarcinoma cell line and was named neurosin (Yamashiro et al., 1997), and from brains of Alzheimer's patients and was named zyme. According to the new official nomenclature for the kallikrein genes, protease M/zyme/neurosin is now named human kallikrein 6 (Diamandis et al., 2000a).

The physiological function(s) of hK6 have not been elucidated. hK6 is most closely related by sequence to human kallikreins, including the prostate-specific antigen (PSA/hK3) (Diamandis, 2000) and normal epithelial cell-specific 1 gene (NES1/hK10), a novel kallikrein with putative tumor suppressor function (Liu et al., 1996; Goyal et al., 1998). Recent studies have shown that the human kallikrein gene cluster contains at least 15 genes tandemly aligned on chromosomal locus 19q13.3–q13.4 (reviewed in Yousef and Diamandis, 2001). All kallikrein genes share significant homologies at the DNA and protein level and, based on primary sequence features, they encode for serine proteases with either trypsin-like or chymotrypsin-like activity. There is growing evidence that several of the kallikrein genes are related to malignancy (Yousef and Diamandis, 2001; Sotiropoulou et al., 2003). PSA is the best tumor marker currently available for the diagnosis and monitoring of prostate cancer (Stamey et al., 1987). Based on its aberrant expression in tumor cells (Anisowicz et al., 1996) and recent studies, hK6 is a potential circulating biomarker for the diagnosis and monitoring of ovarian cancer (Diamandis et al., 2000b, c, 2003; Tanimoto et al., 2001). In addition, hK6 was proposed to play a role in the pathophysiology of Alzheimer's disease (Little et al., 1997) and may be exploited as a biomarker of this condition (Diamandis et al., 2000d; Mitsui et al., 2002). More recently, it was shown that hK6 cleaves myelin (Bernett et al., 2002), a protein implicated in multiple sclerosis and demyelinating diseases. Interestingly, hK6 seems to play a significant role in physiological alpha-synuclein degradation and may be involved in the pathogenesis of Parkinson's disease and other synucleinopathies (Iwata et al., 2003).

The hK6 protein consists of 244 amino acid residues and contains pre- and pro-NH_2-terminal signal peptides with a hydrophobic signal and an activation peptide preceding the mature protein of 223 amino acid residues (Anisowicz et al., 1996). Recently, recombinant pro-hK6 has been obtained from transfected embryonic kidney cells and its substrate specificity has been studied (Magklara et al., 2003). The crystal structures of mature hk6

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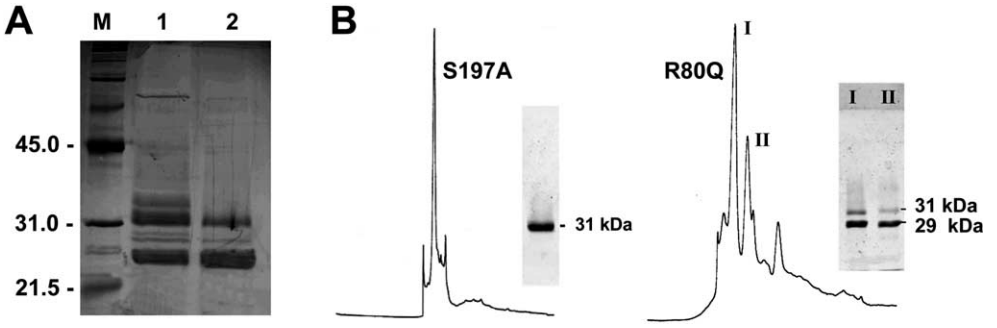

Figure 1 Expression and purification of recombinant pro-hK6 and its mutant forms S197A and R80Q.
Forty-eight hours after induction, the protein present in the yeast culture supernatant was purified by hydrophobic interaction chromatography followed by anion-exchange FPLC. (A) Wild-type pro-hK6 before (lane 1) and after (lane 2) FPLC purification. Samples containing 2 μg of total protein were resolved by SDS-PAGE on a 15% polyacrylamide gel and silver-stained; molecular size markers are shown on the left in kDa. (B) FPLC chromatograms of purified recombinant pro-hK6 mutants S197A and R80Q. Analysis of the eluted fractions by SDS-PAGE and the apparent molecular sizes are shown in the inset.

enzyme (Bernett et al., 2002) and the proenzyme (Gomis-Rüth et al., 2002) have been reported.

The joint consideration of the activation cleavage points of human kallikreins and their trypsin-like activity suggest that a mechanism of autoactivation may occur. In the present study, recombinant pro-hK6 and selected point-mutants were overexpressed in the methylotrophic yeast *Pichia pastoris* to investigate the mechanism of zymogen activation and enzyme degradation. An initial characterization of the enzymatic activity of hk6, both on synthetic and protein substrates, and a survey of the effect of serine protease inhibitors on hk6 activity are also presented.

Results

Overexpression and purification of wild-type pro-hK6

The cDNA sequence encoding pro-hK6 (A^{16}-K^{244}; the sequential numbering of hK6 is used throughout, with Met^{1} and A^{16} corresponding to the N-terminus of the pre-pro-enzyme and the pro-enzyme, respectively) was cloned into the pPCI9 vector in frame with the yeast α-MF. The *Pichia pastoris* recognition signal (Leu-Glu-Lys-Arg-X-) designed for proteolytic processing was included at the N-terminus. The KM71 *P. pastoris* strain was stably transformed and an overexpressing clone was selected and propagated. The recombinant protein was partially purified from the yeast culture supernatant by hydrophobic interaction chromatography and subsequently purified to homogeneity by anion-exchange FPLC, the final yield of purified protein being 15 mg/l of yeast culture. SDS-PAGE analysis followed by silver staining allowed the detection of two major bands of 31 and 25 kDa in the purified fraction (Figure 1A), and Western blot analysis confirmed that both arise fron pro-hK6 (not shown). The N-terminal sequence of the 31 kDa band corresponded to pro-hK6 (A^{16}EEQNKLV-), as designed, while that of the 25 kDa band corresponded to an internal sequence of hK6 (E^{81}SSQEQSS-), indicating cleavage at the internal bond R^{80}-E^{81}, as previously described (Bernett et al., 2002; Magklara et al., 2003).

When the protein was tested *in vitro* for serine protease activity using BAEE as substrate, a low but detectable enzymatic activity was observed for non-activated pro-hK6, suggesting that part of the proenzyme had already undergone activation. Activity increased initially by 4-fold when the zymogen was activated with Lys C at a hK6:LysC molar ratio of 7.5:1, to rapidly decrease and completely disappear within 20–30 min. At the end of this time only the 25 kDa band remained visible, confirming the transformation of one species into the other along an autoinactivation process.

Production and analysis of mutant forms of pro-hK6

Three mutant forms of the hK6 zymogen were designed to overcome the instability problem of wild-type pro-hK6, and with the aim of obtaining samples suitable for structural and functional analysis: an 'inactive' form, in which the catalytic serine 197 was mutated to an alanine (pro-hK6 S197A), a 'stable' mutant, in which the inactivation point detected in the wild-type was changed to a glutamine (pro-hK6 R80Q), and a third one which was produced only for crystallographic purposes (Gomis-Rüth et al., 2002) and contained three point mutations at R80Q, N134Q (designed to eliminate the putative glycosylation site), and Q19R (located at the activation peptide region and a product of PCR cloning). All mutants were purified to homogeneity as described for the wild-type protein. The chromatograms of the final purification step of pro-hK6 S197A and pro-hK6 R80Q are shown in Figure 1B, together with an electrophoretic analysis of their purity.

The purified S197A mutant appeared as a single band at 31 kDa, which did not evolve to generate lower molecular weight species. Its molecular mass was determined by MALDI-TOF to be 27,413 Da and its N-terminal sequence analysis yielded A^{16}EEQNKLV- (Table 1), which is consistent with the sequence predicted for the pro-enzyme. As expected, the S197A mutant form of pro-hK6 displayed no proteolytic activity after being treated with Lys C. Purification of the R80Q mutant form of hK6 yielded two major peaks (I and II in Figure 1B), each containing two bands at 31 and 29 kDa. Both bands of peak I displayed the N-terminal sequence N^{20}KLVHGGP-, which corresponds to pro-hK6 partially truncated at its pro-

Table 1 Molecular masses and NH_2-terminal sequences of recombinant pro-hK6 proteins.

Expressed protein	N-terminal sequence	Molecular mass (Da)			Active before LysC activation	Active after LysC activation	Glycosylated
		Expected	Measured by reducing SDS-PAGE	Measured by MALDI-TOF			
pro-hK6 (S197A)	A^{16}EEQNKL	25 183.5	31 000	27 413±250	No	No	Yes
pro-hK6 (R80Q)	N^{20}KLVHGG	24 714.0	31 000		No	Yes	Yes
Peak I	N^{20}KLVHGG	24 714.0	29 000		No	Yes	No
pro-hK6 (R80Q)	L^{22}VHGGPC	24 471.7	31 000		Yes	Yes	Yes
Peak II	L^{22}VHGGPC	24 471.7	29 000	24 896±300	Yes	Yes	No

Molecular masses were determined by SDS-PAGE and by MALDI-TOF mass spectrometry. N-terminal sequences were determined experimentally as described in Materials and methods. Deglycosylation with *N*-glycosidase F, activation with Lys C, and determination of the enzymatic activity are also described in Materials and methods. A decrease in the apparent molecular mass of glycosylated proteins was observed upon deglycosylation.

region, whereas the sequence of both bands of peak II was L^{22}VHGGPC-, which corresponds to the fully active/mature enzyme. The partially truncated form of pro-hK6 (N^{20}KLVHGGP-) was found to be completely inactive, although it could be fully activated upon addition of Lys C (Table 1). This indicates that the two residues N^{20}K- of the activation peptide are sufficient to keep the enzyme inactive. In addition, it demonstrates the importance of cleaving the right bond in the process of protease activation, as seen for other serine proteases (Gomis-Rüth et al., 1998).

The fact that pro-hK6 R80Q was found both as a zymogen with a truncated pro-region (N^{20}KLVHGGPC-) and as an active enzyme (L^{22}VHGGPC-), while the inactive mutant pro-hK6 S197A retained the complete activating peptide sequence (A^{16}EEQNKL^{22}VH-), indicates that the observed partial and/or complete processing of the pro-region is not an artifact produced by the expression/purification system but is due to the enzyme itself, confirming that hK6 displays an autoactivation ability. Similarly, a fragment corresponding to E^{81}SSQEQ- was not produced in either of the expressed mutants, proving that cleavage at R^{80} is also a product of the autocatalytic activity of wild-type hK6 and that the mutation R80Q protects the enzyme from an auto-degradation process.

Treatment with *N*-glycosidase F showed that the 31 kDa band observed in both peaks of mutant R80Q in Figure 1B corresponds to a glycosylated form, which accounts for a portion of the total protein (Figure 2A). After treatment with *N*-glycosidase F for 24 hours at 37°C, a band at 22 kDa appears as a subproduct of the active enzyme (peak II; see the far right lane in Figure 2A), suggesting that a minor degree of autolysis is still present. In order to determine if a secondary autodegradation point is present, an aliquot of highly pure triple mutant previously purified for crystallographic purposes (pro-hK6 Q19R/R80Q/N134Q) was left at room temperature for three days. Subsequent N-terminal analysis of the sample yielded three clear sequences. The first two (N^{20}KLVHG and L^{22}VHGGP) were expected and support the autoactivation process described. The third sequence (A^{92}VIHPDY) corresponds to the amino acid sequence after R^{91}, and indicates that indeed a secondary autoinactivation point is present in the molecule.

The abnormal electrophoretic mobility of wild type pro-hK6 (Figure 1A) and its mutants (Figures 1B and 2A) has also been observed for other kallikreins, like PSA (Takayama et al., 1997; Habeck et al., 2001). To explain the reason for the anomalously high apparent molecular mass of hK6, mutants S197A and Q19R-R80Q-N134Q were analyzed by SDS-PAGE electrophoresis under reducing and non-reducing conditions and, in the latter case, also treated with *N*-glycosidase F. In the presence of β-mercaptoethanol the electrophoretic mobility of

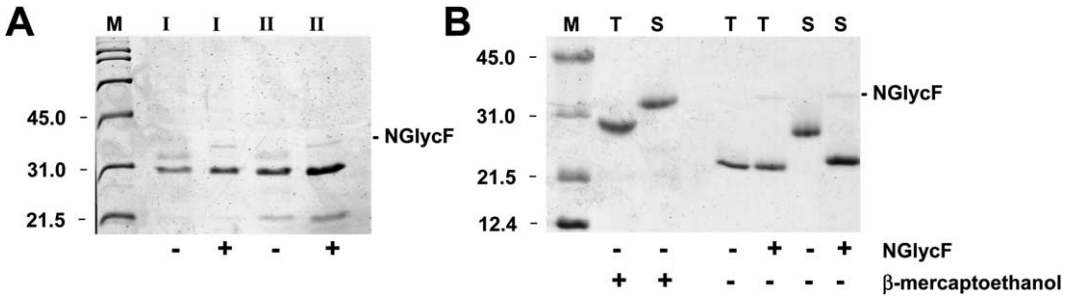

Figure 2 Deglycosylation and electrophoretic behavior of pro-hK6 mutants.
(A) pro-hK6 R80Q mutant. Peaks I and II described in Figure 2 were analyzed, both treated or not treated with *N*-glycosidase F as indicated below the gel. (B) Deglycosylation and analysis of the electrophoretic behavior of pro-hK6 Q19R-R80Q-N134Q mutant (labeled T for Triple mutant) and pro-hK6 S197A mutant (labeled S). Both mutants were treated or not with *N*-glycosidase F and were analyzed with or without added β-mercaptoethanol in the loading buffer. The presence of β-mercatpoethanol and/or *N*-glycosidade F is indicated below the gels, whereas the sample analyzed is indicated at the top of the lanes. Molecular size markers are shown on the left in kDa and the electropohoretc migration of *N*-glycosidase F is indicated on the right side of the gels.

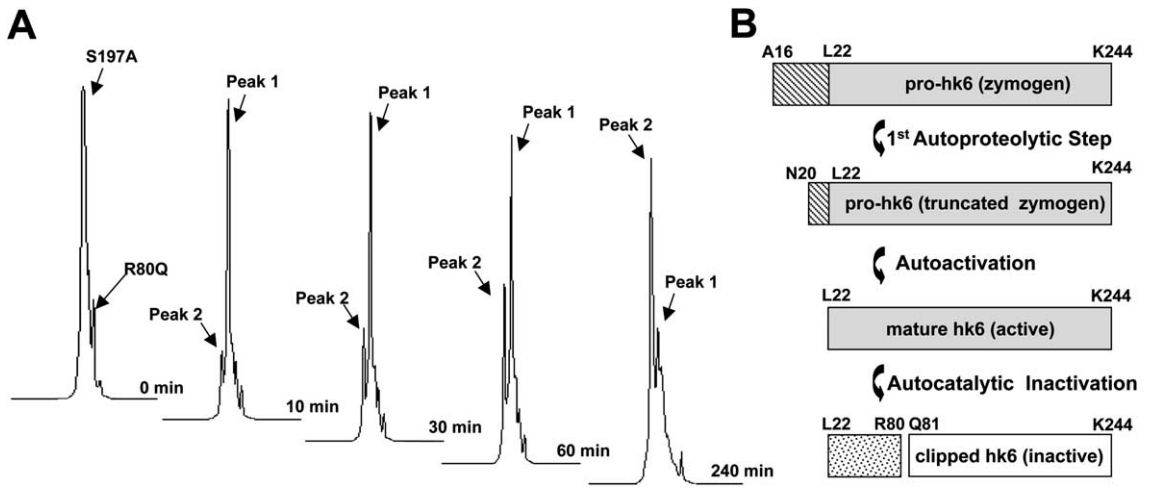

Figure 3 Mechanism of autoactivation and autocatalytic inactivation of pro-hK6.
(A) Time-dependence for the autoproteolysis of recombinant pro-hK6. The enzymatically inactive S197A mutant form of pro-hK6 was used as a substrate for active hK6 R80Q at a substrate:enzyme ratio of 20:1. Shown are the chromatograms of the resulting proteolytic fragments at different digestion times. Peak 1 contains the N-terminal sequence A^{16}EEQNKLV- (zymogen) and peak 2 contains sequences N^{20}KLVHGGP- (truncated zymogen), L^{22}VHGGPCW- (mature enzyme) and E^{81}SSQEQSS- (clipped enzyme). (B) Schematic representation of the autoproteolytic steps leading to maturation of pro-hK6 and the subsequent inactivation of the mature enzyme by self-cleavage at the internal bond R^{80}-Q^{81}.

these two mutants is slower than expected from their molecular masses (Figure 2B, lanes 1–2), but it is consistent with their calculated mass under non-reducing conditions (Figure 2B, lanes 3–6). The S197Q mutant, which contains an intact glycosylation target (N^{134}), is indeed glycosylated, whereas the triple mutant bearing a N134Q mutation has the same mobility before and after treatment with *N*-glycosidase F. Table 1 shows a summary of the expected and observed molecular masses for the S197A and R80Q mutants that confirms the above mentioned discrepancies.

Mechanism of autoactivation

To confirm the process of autoactivation/autoinactivation proposed above, an autoproteolysis experiment was designed in which the proteolytically inactive S197A mutant form of the pro-hK6 was used as a substrate for the stabilized mature enzyme (R80Q mutant; Figure 3A). At the indicated times, reaction samples were removed and the proteolytic products were analyzed by reverse-phase HPLC and N-terminal sequencing. As the intensity of peak 1 in the chromatogram (which corresponds to the S197A substrate) gradually decreases, a second peak containing the N-terminal sequences N^{20}KLVHGGP-, L^{22}VHGGPCW- and E^{81}SSQEQSS gradually increases. Although the multi-component peak 2 could not be better resolved by HPLC, an apparent increase in the yield of sequences L^{22}VHGGPCW- and E^{81}SSQEQSS was observed at increasing incubation times. This indicates that the enzyme is able to autoactivate via a two-step process in which the bond Q^{19}-N^{20} in the activation peptide is recognized and cleaved first, followed by the removal of the N^{20}K dipeptide to yield an active enzyme. Since the main end product of the autoproteolysis experiment is the E^{81}-K^{244} internal fragment of hK6, as confirmed by the absence of any other degradation products by mass spectrometry analyses (data not shown), it appears that R^{80} is the main site for self-cleavage in mature hK6 and that the secondary autoinactivation event that would yield the sequence A^{92}VIHPDY after cleavage at R^{91} only takes place in the absence of the main autoinactivation target. Based on this data, a scheme for the complete process can be proposed (Figure 3B). Overall, these results indicate that hK6 recognizes R, K and also Q as P1 residues although its substrate specificity might be restricted by the presence of specific amino acids in the P2, P3 and/or P1′ positions.

Substitution of R^{80} with Q stabilizes the enzymatic activity of hK6

After activation of wild-type pro-hK6 with lysil endopeptidase (Lys C), N-terminal analysis of the generated products indicated that Lys C cleaves the K^{21}-L^{22} bond. However, the enzymatic activity of hK6, as measured by following the hydrolysis of BAEE, although initially increased upon treatment with Lys C, could not reach its maximun and showed a continuous decrease 10 min after activation and during prolonged incubation at 25°C. Both the autoinactivation mechanism described above and the proteolytic processing of mature hK6 by Lys C likely account for this loss in activity, since the rate of inactivation was also dependent on the concentration of Lys C (data not shown). When stored at room temperature, wild-type pro-hK6 exhibited a low intrinsic proteolytic activity, which rapidly disappeared due to autoinactivation, whereas the activity of the pro-hK6 R80Q mutant reached a maximum after activation and remained stable after four hours of storage at room temperature (data not shown). These results indicate that a single amino acid substitution at R^{80} is sufficient to prevent inactivation *via* autoproteolysis and leads to stabilization of mature hK6, while it does not seem to alter its enzymatic properties. On the other hand, after storage of recombinant pro-hK6 R80Q in solution for a period of several weeks at −20°C, complete conversion into

mature hK6 was obtained. This fully autoactivated and stable enzyme was used in all activity assays described below.

Enzymatic activity of hK6 on synthetic and protein substrates and effect of inhibitors

Consistently with a trypsin-like activity, recombinant hK6 cleaved the N^{α}-benzoyl-L-arginine ethyl ester (BAEE) substrate, although its activity corresponded to only 64% of the activity of an equimolar amount of TPCK-treated bovine trypsin. Interestingly, hK6 did not hydrolyze the N^{α}-tosyl-L-arginine methyl ester (TAME) substrate, also typical for trypsin, suggesting that hK6 displays a more restricted substrate specificity. No activity was observed against chymotrypsin-, plasmin-, urokinase- or elastase-specific substrates (not shown). The proteolytic activity of hK6 was weakly inhibited by known macromolecular inhibitors of serine proteases, such as soybean trypsin inhibitor (STI), Bowman-Birk inhibitor (BBI), and bovine pancreatic trypsin inhibitor (BPTI). The α-aryl-lactone inhibitor (3-[4-(guanidino-methyl)phenyl]-6-methylidene-tetra-hydro-2-pyranone) (Sotiropoulou et al., 2003) was effective only at high concentrations, while leupeptin, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) produced no significant effect (not shown). Therefore, a putative specific inhibitor of hK6 remains to be identified.

Zymographic analysis of hK6 action on protein substrates revealed that gelatin was degraded by mature hK6, although with markedly lower efficiency when compared to TPCK-treated bovine trypsin, while casein was barely cleaved. Hydrolysis of prothymosin-α by hK6 yielded only one of the four major peaks generated by trypsin, as analyzed by reverse phase HPLC. Similarly, hK6 had no effect on native urokinase plasminogen activator (uPA) or on reduced and carboxymethylated uPA substrate (results not shown). These results, together with those obtained with synthetic substrates, suggest that hK6 has a restricted specificity on protein substrates.

Interestingly, human plasminogen (Plg) was efficiently hydrolyzed by mature hK6. As shown in Figure 4, plasminogen was cleaved into three major bands of about 60, 55 and 44 kDa. The 55 and 60 kDa bands both displayed the N-terminal sequences K^{97}VYLSECKTG- and V^{98}YLSECKTG-, identical to those originally reported for the angiogenesis inhibitor angiostatin, generated by elastase (O'Reilly et al., 1994; Dong et al., 1997). The 44 and 38 kDa bands both displayed the N-terminal sequence V^{461}VAPPPVVLL-, that corresponds to residues 461–470 of the prepro-plasminogen at the junction between kringle domain 4 and kringle domain 5 (Cao et al., 1996) and results from the cleavage of the S^{460}-V^{461} peptide bond in prepro-plasminogen. PSA was also shown to produce biologically active angiostatin by cleaving prepro-plasminogen at the bond E^{458}-A^{459} (Heidtmann et al., 1999), two amino acids upstream from the cleavage site identified here for hK6. These results indicate a specificity of the cleavage site among different kallikreins, since both PSA and hK6 recognize a unique cleavage site between kringle domain 4 and kringle domain 5 completely distinct from other proteases known to generate angiostatin from plasminogen.

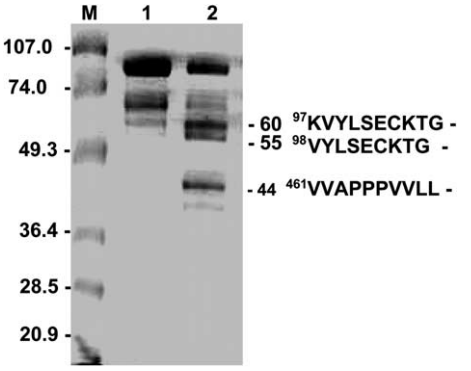

Figure 4 Hydrolysis of plasminogen by mature hK6 R80Q and production of angiostatin.
Plasminogen isolated from human plasma was incubated in the absence (lane 1) or presence (lane 2) of mature hK6 R80Q at a molar ratio of enzyme:substrate of 1:65 for 24 hours at room temperature. The resulting proteolytic fragments were analyzed by SDS-PAGE on a 12% acrylamide gel and stained with Coomassie Brilliant Blue R-250. The N-terminal sequences of plasminogen fragments were determined experimentally and are indicated on the right. Molecular weight markers are shown on the left in kDa.

Discussion

Purified recombinant pro-hK6 displays an intrinsic enzymatic activity that leads to autoactivation and subsequent autoproteolytic processing at the internal residue R^{80} that renders the enzyme inactive. The mechanism of autoproteolysis of pro-hK6 was investigated by analysis of the effect of the stable mutant R80Q on the inactive mutant S197A. Although it was hypothesized that autoactivation does not seem very likely to occur in pro-hK6, since it displays a low k_{cat}/K_m value for lysine in the P1 position (Bernett et al., 2002), mutant R80Q suffers an autoactivation process similar to the wild-type protein, while the S197A mutant maintains the N-terminal sequence of the zymogen. In mutant R80Q, however, autoinactivation is avoided by substituting the original R^{80} target. Here we show that autoactivation is likely to take place through a two-step mechanism, with a first proteolytic step occurring after Q^{19} that does not yet render an active enzyme, followed by a second cleavage that hydrolyzes the peptide bond after K^{21}. Similarly to hK6, hK2 has autocatalytic activity because it can cleave pro-hK2 at Arg^{-1}, releasing the propeptide (Mikolajczyk et al., 1997). In addition, 20–30% of PSA in seminal fluid is typically cleaved between residues 85–86 (R-F), 145–146 (K-K) or 182–183 (K-S) (Christensson et al., 1990). Thus, hK6 shares common features with known kallikreins.

Fully autoactivated mature and stabilized hK6 (R80Q mutant) displayed a trypsin-like activity. However, and contrary to the observations with other known serine proteases, gelatin, casein, prothymosin-α and uPA as well as several synthetic peptide substrates were not efficiently hydrolyzed by active hK6. Although it must be mentioned here that in a previously published report (Magklara et al., 2003) hK6 was found to be efficient in

the digestion of casein, collagen or fibrinogen, it appears that hK6 may have a rather restricted substrate specificity. Self-cleavage of hK6 and hydrolysis of human plasminogen indicated that hK6 displays amidolytic activity for R-*X*, K-*X*, Q-*X* and S-*X* bonds on protein substrates. This enlarges the range of susceptible bonds beyond Arg and Lys (Bernett et al., 2002; Magklara et al., 2003) and suggests that its specificity might, as well, be restricted by the presence of specific amino acids in the P2, P3 and/or P1′ positions.

Glycosylation is a key feature shared by several human kallikreins. By site-directed mutagenesis, we have shown that glycosylation of hK6 occurs at N^{134}. This glycosylation site was hypothesized not to significantly affect the specificity and function of the enzyme due to its distal location from the active site (Bernett et al., 2002). Indeed, we found that the glycosylated and non-glycosylated recombinant proteins displayed the same levels of enzymatic activity (not shown). In this regard, it is interesting to note that to obtain an accurate estimation of the size of the sugar chains added it is advisable to perform an SDS-PAGE analysis of the protein in non-reducing conditions and with and without added glycosidases, as shown in Figure 2B.

Secreted serine proteases are expected to act on extracellular targets, such as components of the extracellular matrix, growth factors or surface receptors. Proteases like matrix metalloproteinases and cysteine proteinases are usually overexpressed in metastatic tumor cells and are considered to promote tumor invasion and metastasis. However, certain kallikreins including PSA (hK3), hK6 and NES1 (hK10) exhibit a reduced or absent expression in breast and prostate metastatic tumor cells compared to corresponding normal cells (Diamandis, 2000; Anisowicz et al., 1996; Liu et al., 1996; Goyal et al., 1998) and may have a tumor suppressor function. For example, although PSA levels are elevated in the serum of prostate cancer patients and their rising levels represent a marker of disease progression (Stamey et al., 1987), its expression is reduced in malignant cells as compared to normal cells (Diamandis, 2000). In addition, anti-angiogenic properties of PSA were described by virtue of its ability to inhibit endothelial cell responses to pro-angiogenic factors bFGF and VEGF. The anti-angiogenic and antitumor effects of PSA were shown to result from its ability to produce biologically active angiostatin from plasminogen (Fortier et al., 1999; Heidtmann et al., 1999). We have shown that human kallikrein 6 can also generate angiostatin from plasminogen and may thus have similar antiangiogenic effects.

Several proteolytic enzymes produced by tumor-activated stromal cells, such as macrophage metalloelastase (Dong et al., 1997) and matrix metalloproteinases like stromelysin-1 (MMP-3; Lijnen et al., 1998), MMP7 and MMP9 (Patterson et al., 1997), were shown to generate angiostatin from plasminogen *in vitro*. *In vivo*, angiostatin is produced by tumor cells and, therefore, tumor proteases are likely to be involved in its generation (Folkman, 1995). Our data suggest that hK6 is a self-regulated protease produced by primary epithelial tumor cells, which is able to release angiostatin from plasminogen. The fact that both PSA and hK6 can release the angiogenesis inhibitor *via* specific proteolysis of plasminogen, indicates that distinct kallikreins may be responsible for the generation of angiostatin *in vivo*, and may play an important role in the regulation of the angiogenic balance during tumor growth and dissemination.

Materials and methods

Expression constructs

The cDNA sequence (nt 291–977) (GenBank™ accession number: U62801) encoding human pro-hK6 (A^{16}-K^{244}) was amplified by PCR and cloned into the *Pichia pastoris* expression vector pPCI9 in frame with the yeast α-mating factor (α-MF; Invitrogen, Carlsbad, USA). For proteolytic processing of the fusion protein, the *Pichia pastoris* recognition signal (Leu-Glu-Lys-Arg-X) was designed between the carboxy terminal of the α-MF and the N-terminal of pro-hK6. To facilitate cloning, *Xho*I and *Eco*RI restriction sites were introduced at the 5′- and 3′-end, respectively. The following primers were used for PCR amplification: 5′-GGCTCGAGAAAAGAGCAGAGGAGCAG-3′ (forward) and 5′-GTCGGAATTCAGGGTCA CTTGGCCTG-3′ (reverse). The PCR product was digested with *Xho*I and *Eco*RI and ligated into the pPIC9 vector between the 5′-promoter and the 3′-terminator of the AOX1 gene. All DNA manipulations were carried out by standard methods using the *E. coli* strain DH5α (Sambrook et al., 1989). Restriction endonucleases and *Taq* DNA polymerase were purchased from Gibco BRL, Carlsbad, USA, T4 DNA ligase and *Vent* polymerase from New England Biolabs, Beverley, USA, ultrapure 2′-deoxynucleotide, 5′-triphosphates (dNTPs) from Pharmacia, Uppsala, Sweden, and *N*-glycosidase F from Boehringer Mannheim, Mannheim, Germany. Culture media for *E. coli* were purchased from Difco, Sparks, USA, and for *Pichia pastoris* from Sigma, St. Louis, USA.

Expression and purification of recombinant pro-hK6

The *Pichia pastoris* strain KM71 was stably transformed using 10 μg of plasmid (pPIC9/prohK6 expression construct) previously linearized with *Sal*I, according to the spheroplast method. For selection of overexpressing transformants, colonies were grown in 15 ml of BMGY growth medium (1% yeast extract, 2% peptone, 90 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin and 1% glycerol) at 30°C for 2 days. Expression of the recombinant protein was induced when cell density reached 16–20 absorbance units at 600 nm. Cells were collected by centrifugation at 1500 *g* and gently resuspended in 3 ml of BMMY medium (BMGY medium containing 1% methanol instead of 1% glycerol) and cultured for two additional days. Stably transformed clones overexpressing pro-hK6 were selected and propagated. Recombinant pro-hK6 was purified from the culture supernatant by hydrophobic interaction chromatography on a Butyl-Toyopearl 650 M column (Tosoh, Tokyo, Japan) and eluted with a decreasing gradient of ammonium sulfate. Elution of recombinant protein was monitored by SDS-PAGE and eluted fractions were pooled and dialyzed against 10 mM Tris-HCl pH 8.0. Recombinant pro-hK6 was purified to homogeneity by preparative FPLC (Pharmacia LCC-500) on an anion-exchange column TSK-DEAE 5PW (Tosoh) and eluted with a linear gradient of ammonium acetate in 10 mM Tris-HCl pH 8.0, except for pro-hK6 (R80Q), which was eluted with the same buffer at pH 7.7.

Site-directed mutagenesis

The cDNA sequence encoding pro-hK6 was cloned into the pBlueScript *SK* vector (Stratagene, La Jolla, USA) and used as

a template for PCR amplification. Site-specific mutations were introduced by a two-step PCR method using the following internal primers: S197A mutant: 5′-GATGCTGGGGGTCCGCTGGTATGTGGAGACCACCTCCG-3′ (forward) and 5′-ACCAGCGGACCCCCAGCATCACCCTGGCAGGA-3′ (reverse); R80Q mutant: 5′-ACCCTTCGGCAACAGGAGAGTTCCCAGGAGCAGAG-3′ (forward) and 5′-CTCCTGTTGCCGAAGGTTATGCTTCCCCAGGAAGACC-3′ (reverse); R80Q/N134Q double mutant: 5′-GCTCAGCCCAAACCACCAGCTGCCACA-3′ (forward) and 5′-GGTGGTTTGGGCTGAGCAGTCCCTC-3′ (reverse). All constructs were sequenced in order to confirm the presence of the desired mutations and the lack of undesired mutations randomly introduced by PCR.

Western blotting

After transference to PDVF membranes, proteins were identified using a rabbit polyclonal antibody raised against recombinant pro-hK6 (kindly provided by Dr. E. P. Diamandis, Mount Sinai Hospital, Toronto). Anti-rabbit horseraddish peroxidase-linked IgG was used as secondary antibody. Immunoreactive proteins were detected with an enhanced chemilumine-scence kit (Amersham, Uppsala, Sweden). The primary antibody was used at a dilution of 1:1000, while the secondary antibody was used at a dilution of 1:2500.

Deglycosylation of pro-hK6

Deglycosylation was performed in a 40 μl reaction containing 4 μg of each recombinant protein and 4 units of *N*-glycosidase F (Boehringer Mannheim) in 50 mM Tris-HCl pH 7.5. The mixture was incubated at 37°C for 24 h and the reaction products were resolved by SDS-PAGE. Deglycosylation did not require previous denaturation of the recombinant proteins.

Activity assays

Recombinant pro-hK6 was treated with lysil endoprotease (Lys C; Wako, Tokyo, Japan) at a molar ratio of pro-hK6:Lys C of 75:1 at 25°C. At given times, aliquots were removed and the proteolytic activity was assayed in 67 mM Na_2HPO_4 pH 7.6 using the *N*-α-benzoyl-L-arginine-ethyl-ester (BAEE) substrate and monitored spectrophotometrically at 254 nm. The enzymatic activity against *N*-α-tosyl-arginine-methyl-ester (TAME) was assayed in 40 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.1 and monitored at 247 nm. TPCK-treated bovine trypsin (Worthington, Lakewood, USA) was assayed for comparison. For inhibition assays, 2 μg of pro-hK6 were activated with Lys C for 2 min at a 7.5:1 molar ratio. Inhibitors were added at molar ratios varying from 1:1 to 1:8 and the remaining proteolytic activity was measured using the BAEE substrate. Chromogenic substrates were purchased from Sigma or Chromogenix (Milano, Italy) and inhibitors from Sigma.

Cleavage of protein substrates

Mature hK6 R80Q mutant was used for cleavage of protease substrates. The following protein substrates were cleaved: inactive recombinant pro-hK6 S197A mutant, human prothymosin *alpha* (Thymoorgan, Vienenburg, Germany), urokinase isolated from human kidney cells (Fluka, St. Louis, USA) and plasminogen isolated from human plasma (Sigma). Cleavage of substrates by trypsin was monitored as a control. Mature hK6 was incubated with the pro-hK6 S197A substrate in 10 mM Tris-HCl pH 8.0 at room temperature at a substrate:enzyme molar ratio of 20:1. Aliquots were removed at given times and the reaction was terminated by adding 0.05% trifluoroacetic acid (TFA). The resulting proteolytic fragments were analyzed by reverse-phase HPLC (Waters, Milford, USA) using a C_4 column eluted with a linear gradient of water:acetonitrile in 0.1% TFA and monitored at 214 nm. 30 μg of human plasminogen were incubated with 500 ng of mature hK6 in 30 μl of 10 mM Tris-HCl pH 8.0 at 37°C. Aliquots were removed at the indicated time points and the reaction was stopped with reducing SDS-PAGE sample buffer. Cleavage products were resolved on 12% polyacrylamide gels and silver-stained or blotted onto Immobilon-P membrane (Millipore, Bedford, USA) for N-terminal sequencing. Similar procedures were used for the rest of protein substrates with minor adjustments in the molar rations and incubation times.

Mass spectrometry

Samples of recombinant proteins and HPLC elution fractions were analyzed by mass spectrometry using a matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) spectrometer (Biflex with Reflectron, Bruker, Bremen, Germany). 1 μl of each sample (~50 pmol) was mixed with 1 μl of 50% synapinic acid as a matrix and loaded. A method for signal improvement (Botting, 2000) was also applied when needed.

Protein sequencing

For N-terminal sequence determinations, purified proteins were blotted onto polyvinylidene difluoride (PDVF) membrane (Millipore), and analysis was performed by automated Edman degradation using a Beckman LF3000 Protein Sequencer (Beckman, Fullerton, USA).

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