the FEBS Journal

Mutant recombinant serpins as highly specific inhibitors of human kallikrein 14

Loyse M. Felber[1,2], Christoph Kündig[1,2], Carla A. Borgoño[3], Jair R. Chagas[4], Andrea Tasinato[1], Patrice Jichlinski[1], Christian M. Gygi[1], Hans-Jürg Leisinger[1], Eleftherios P. Diamandis[3], David Deperthes[1,2] and Sylvain M. Cloutier[1,2]

1 Urology Research Unit, Department of Urology, CHUV, Epalinges, Switzerland
2 Medical Discovery SA, Epalinges, Switzerland
3 Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada
4 Centro Interdisciplinar de Investigacao Bioquimica, Universidade de Mogi das Cruzes, Brazil

Keywords
inhibitor; kallikrein; protease; serpin

Correspondence
D. Deperthes, Urology Research Unit/Medical Discovery SA, Biopôle, Ch. Croisettes 22, CH-1066 Epalinges, Switzerland
Fax: +41 21 6547133
Tel: +41 21 6547130
E-mail: david.deperthes@med-discovery.com

(Received 15 September 2005, revised 1 March 2006, accepted 3 April 2006)

doi:10.1111/j.1742-4658.2006.05257.x

The reactive center loop (RCL) of serpins plays an essential role in the inhibition mechanism acting as a substrate for their target proteases. Changes within the RCL sequence modulate the specificity and reactivity of the serpin molecule. Recently, we reported the construction of α1-antichymotrypsin (ACT) variants with high specificity towards human kallikrein 2 (hK2) [Cloutier SM, Kündig C, Felber LM, Fattah OM, Chagas JR, Gygi CM, Jichlinski P, Leisinger HJ & Deperthes D (2004) *Eur J Biochem* **271**, 607–613] by changing amino acids surrounding the scissile bond of the RCL and obtained specific inhibitors towards hK2. Based on this approach, we developed highly specific recombinant inhibitors of human kallikrein 14 (hK14), a protease correlated with increased aggressiveness of prostate and breast cancers. In addition to the RCL permutation with hK14 phage display-selected substrates E8 (LQRAI) and G9 (TVDYA) [Felber LM, Borgoño CA, Cloutier SM, Kündig C, Kishi T, Chagas JR, Jichlinski P, Gygi CM, Leisinger HJ, Diamandis EP & Deperthes D (2005) *Biol Chem* **386**, 291–298], we studied the importance of the scaffold, serpins α1-antitrypsin (AAT) or ACT, to confer inhibitory specificity. All four resulting serpin variants ACT_{E8}, ACT_{G9}, AAT_{E8} and AAT_{G9} showed hK14 inhibitory activity and were able to form covalent complex with hK14. ACT inhibitors formed more stable complexes with hK14 than AAT variants. Whereas E8-based inhibitors demonstrated a rather relaxed specificity reacting with various proteases with trypsin-like activity including several human kallikreins, the two serpins variants containing the G9 sequence showed a very high selectivity for hK14. Such specific inhibitors might prove useful to elucidate the biological role of hK14 and/or its implication in cancer.

The human tissue kallikrein family is composed of 15 secreted serine proteases (hK), encoded by 15 highly similar genes (*KLK*) in terms of structure and regulation [1–4]. The best studied member, hK3 [also known as prostate-specific antigen (PSA)] is a valuable marker for prostate cancer diagnosis and monitoring. More recently, hK2 has also emerged as a promising combined biomarker for prostatic carcinoma, especially in

Abbreviations
AAT, α_1-antitrypsin; ACT, α_1-antichymotrypsin; AMC, 7-amino-4-methylcoumarin; E, enzyme; hK, kallikrein protein; I, inhibitor; IPTG, isopropyl thio-β-D-galactoside; KLK, kallikrein gene; NaCl/Pi, phosphate-buffered saline; OD, optical density; PSA, prostate-specific antigen; r, recombinant; RCL, reactive center loop; SI, stoichiometry of inhibition.

improving discrimination between prostate cancer and benign prostatic hyperplasia [5–7].

Several genes of the kallikrein family are aberrantly expressed in various cancers [2,3] but especially in hormone-dependent cancers such as prostate, breast [8–10], ovarian [11] or testicular cancers [12]. One gene, *KLK14*, encoding the human kallikrein 14 protein (hK14) is found in various biological fluids and tissues, including central nervous system and in endocrine-related tissues, such as breast, prostate, thyroid and uterus [13,14].

hK14 was identified by ELISA and immunohistochemistry in breast, skin and prostatic tissues, as well as seminal plasma and amniotic fluid [15]. Like several other kallikreins, hK14 is up-regulated by steroid hormones such as androgens [16] and estrogens [15].

hK14 was proposed as a potential new biomarker for breast and ovarian cancers, since elevated serum levels were found in 40 and 65% of patients with these cancers, respectively [15]. Moreover, hK14 expression correlates with poor prognosis in breast [17] and prostate [18] cancers. These findings led us to hypothesize that hK14 may play a role in cancer initiation and/or progression, although its biological function is still unknown.

Recently, we characterized the enzymatic activity of human kallikrein 14 using phage display technology [19] and identified trypsin and chymotrypsin-like activities with a preference for an arginine residue in position P1. Despite this dual activity, hK14 exhibits high specificity towards potential substrates, suggesting targeted biological roles. Several candidate substrates have been identified by bioinformatic analysis, among which are proteins of the extracellular matrix.

One of the strategies to study the involvement of proteases in biological processes includes development of specific inhibitors. Recently, our group described the preparation of specific antiproteases against human kallikrein 2 [20]. A human serpin named alpha 1-antichymotrypsin was used to change its specificity by modifying five amino acids of its reactive center loop, which is the region involved in inhibitor–protease interaction and acts as substrate. The importance of RCL cleaved sequence in protease specificity of serpins is well described in the literature. However, proximal regions of the cleaved site of the inhibitor are also implicated in protease recognition and influence its specificity.

Here, we report the development of hK14-specific inhibitors by modifying the RCL region of two different serpin scaffolds: α_1-antichymotrypsin (ACT) and α_1-antitrypsin (AAT). Two serpins were selected in order to define the importance of the scaffold in the development of new inhibitors. Phage display-selected substrate pentapeptides specific for hK14 [19] were used to replace the scissile bond region of the wild-type serpins. These inhibitors were highly reactive towards hK14 and displayed varying specificities for hK14 and other enzymes, depending on the scaffold.

Results

Design and production of soluble recombinant serpins

To develop inhibitors specific to hK14, we substituted five residues surrounding the scissile bond of rAATwt and rACTwt with two substrate pentapeptides, previously selected by hK14 using phage-display technology [19]. Profiling of hK14 enzymatic activity demonstrated that hK14 has trypsin and chymotrypsin-like activity. We therefore decided to develop inhibitors with two different substrate peptides, E8 and G9, specific for trypsin and chymotrypsin-like activity, respectively. The scissile bond of these substrates was aligned according to the P1-P1′ positions of rAATwt and rACTwt. The RCL regions of the serpin variants are shown in Table 1.

The recombinant serpins were produced as soluble, active proteins and were purified under native conditions from cytoplasmic proteins in a one-step procedure using nickel affinity chromatography. Analysis on SDS/PAGE under reducing conditions revealed a single band for each inhibitor, rAAT and rACT variants, migrating at apparent sizes of 45–50 kDa, corresponding to their molecular weight. All inhibitors were estimated to be more than 95% pure by densitometric analysis, with production yields above 1 $mg{\cdot}L^{-1}$ of culture (data not shown).

Stoichiometry of inhibition, association constants and complex stability

Determination of stoichiometry of inhibition (SI) was performed under physiological conditions of pH and ionic strength. The SI indicates the number of inhibitor molecules required to inhibit one molecule of hK14. Figure 1 shows the determination of SI values (x-intercept) for wild-type serpins and their variants with hK14. We observed that titration curves were linear, even for SI values $>>1$, indicating that the reaction reached completion. The calculated SI values of the serpin variants ranged from $\sim$1–1.5, except for $rAAT_{E8}$ which resulted in an SI of 7.4 (Table 2). Whereas rACTwt did not react with hK14 under the tested conditions, rAATwt was found to be an efficient

Table 1. Comparison of amino acid sequence of the scissile bond region of the reactive serpin loop (RCL) of wild-type AAT, ACT and their variants. Plain type residues are common to wild-type serpin; bold residues correspond to amino acids relocated in RCL of AAT and ACT variants. The scissile bond cleaved by hK14 in substrate peptides is designated by ↓ and putative cleavage sites in serpins are marked by asterisks between P1 and P1′ residues.

Serpin	Selected substrate peptide[a]	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′
AAT_{WT}		L	E	A	I	P	M*	S	I	P	P	E
AAT_{E8}	LQR↓AI	L	E	A	**L**	**Q**	**R***	**A**	I	P	P	E
AAT_{G9}	TVDY↓A	L	E	**T**	**V**	**D**	**Y***	**A**	I	P	P	E
ACT_{WT}		V	K	I	T	L	L*	S	A	L	V	E
ACT_{E8}	LQR↓AI	V	K	I	**L**	**Q**	**R***	**A**	**I**	L	V	E
ACT_{G9}	TVDY↓A	V	K	**T**	**V**	**D**	**Y***	**A**	A	L	V	E

[a]Substrate peptides selected by kallikrein hK14 using a phage-displayed random pentapeptide library [19].

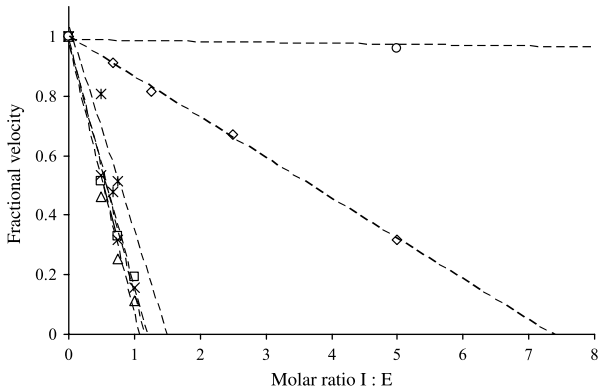

Fig. 1. Stoichiometry of inhibition (SI) of hK14 by rAAT, rACT and their variants. hK14 (2 nM) was incubated with different concentrations (0.5–100 nM) of rAATwt(△), AAT_{E8} (◇), AAT_{G9} (□), rACTwt (○), ACT_{E8} (x) and ACT_{G9} (*) at 37 °C for 4 h in reaction buffer. Residual activities (velocity) of hK14 were obtained by adding 20 μM of fluorescent substrate. Fractional velocity corresponds to the ratio of the velocity of the inhibited enzyme (v_i) to the velocity of the uninhibited control (v_0). SI values were determined using linear regression analysis to extrapolate the $[I]_0/[E]_0$ ratio (i.e. the *x* intercept).

Table 2. Stoichiometry Inhibition (SI) and second-order rate constant (k_a) values for the reaction of rAATwt, rACTwt and their variants with hK14. –, No detectable inhibitory activity.

Inhibitor ($M^{-1}\cdot s^{-1}$)	Selected[a] Substrate peptide	SI	k_a
AAT_{WT}	IPM*SI	1.0	263 000
AAT_{E8}	LQR?AI	7.4	–
AAT_{G9}	TVDY?A	1.2	217 000
ACT_{WT}	TLL*SA	–	–
ACT_{E8}	LQR?AI	1.2	575 000
ACT_{G9}	TVDY?A	1.5	74 000

[a]Substrate peptide selected by kallikrein hK14 using a phage-displayed random pentapeptide library [19] and used to modify the rAATwt and rACTwt.

inhibitor for hK14 with a SI of 1. Substitution of ACT RCL region with hK14 substrate peptides generated inhibitors with high reactivity toward the enzyme. The modification of rAATwt did not increase its reactivity for hK14 (Table 2).

Calculated SI values were consistent with the ratio between cleaved and complexed forms of the serpins after reaction with hK14, as demonstrated by SDS/PAGE analysis (Fig. 2). Inhibitors were incubated with different concentrations of hK14 corresponding to a ratio of inhibitor to protease below, equal and above the SI value. SDS/PAGE analysis showed the formation of covalent complexes with apparent molecular masses consistent with expected values, i.e. the sum of both enzyme and cleaved inhibitor molecular weights. With a $[I]_0/[E]_0$ ratio of 0.6 (ACT_{E8}) and 0.75 (ACT_{G9}), degraded forms of the complex were observed, likely generated by the uncomplexed, free hK14. With this concentration of enzyme, the reaction also produced a fraction of hydrolyzed inhibitor.

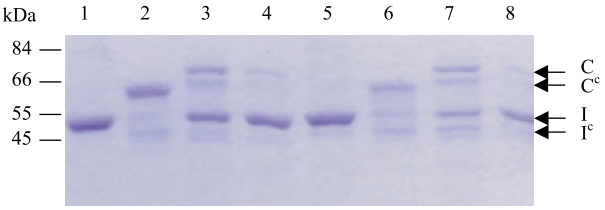

Fig. 2. Complex formation between hK14 and recombinant inhibitors. A constant amount of each ACT variant (1 μg) was incubated for 4 h in reaction buffer without and with different amounts of hK14. Lane 1–4 correspond to ACT_{E8} alone, $ACT_{E8}/hK14 = 0.6$, 1.2 and 2.4, lane 5–8 correspond to ACT_{G9} alone, $ACT_{G9}/hK14 = 0.75$, 1.5 and 3. Samples were heated at 90 °C for 10 min, resolved on a 10% SDS gel under reducing conditions and then visualized by Coomassie blue staining. The position of native inhibitor (I), cleaved inhibitor (I^c), complex (C) and cleaved complex (C^c) are indicated by arrows.

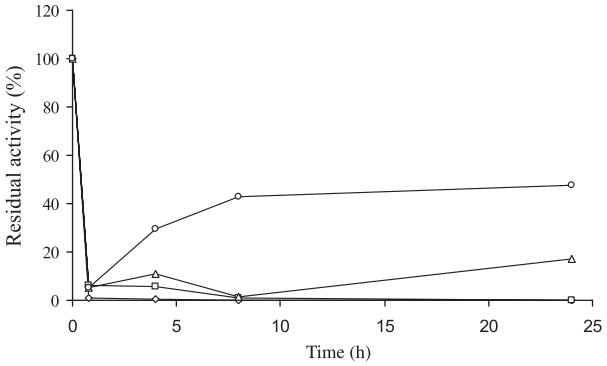

Fig. 3. Stability of complexes between hK14 and recombinant inhibitors over 24 h. Residual activity was measured following complex formation between hK14 and AAT_{E8} (○) ($[I]_o/[E]_o = 14.8$), AAT_{G9} (△) ($[I]_0/[E]_0 = 2.4$), ACT_{E8} (◇) ($[I]_0/[E]_0 = 2.4$) or ACT_{G9} (□) ($[I]_0/[E]_0 = 3$) after 45 min, 4, 8 and 24 h of incubation at 37 °C. Residual activity was normalized to incubated, uninhibited hK14.

Only the $rAAT_{E8}$ variant with an SI value much greater than 1 acted as a substrate for hK14, resulting mainly in accumulation of the cleaved form of the inhibitor rather than formation of the irreversible complex (data not shown). As expected, the presence of intact inhibitor was observed when the ratio $[I]_0/[E]_0$ was above the SI.

ACT complexes were found to be stable for at least 24 h at 37 °C, unlike AAT complexes which degraded after 45 min ($rAAT_{E8}$) and 8 h ($rAAT_{G9}$), resulting in the reappearance of free hK14 and its enzymatic activity (Fig. 3).

Kinetic analysis of the inhibition of hK14 by recombinant serpins was performed under pseudo-first-order conditions using an excess of inhibitor at various molar ratios of hK14. The time-dependent inactivation of the enzyme by the serpin was monitored continuously, following the decrease in the rate of substrate turnover. Progress curves for reactions with different serpin concentrations were fitted to Equation 1 (Experimental procedures) to calculate values describing the rate constant (k_{obs}). Figure 4 shows the concentration dependence of k_{obs} of serpins on hK14 inhibition. The association rate constants (k_a) were determined from the slope of k_{obs} values versus the concentration of hK14 inhibitors. Independent of the inhibitor scaffold (AAT or ACT), the recombinant serpins modified with the E8 substrate showed superior k_a values than their G9 counterparts. Serpins modified with the chymotrypsin-like substrate, $rAAT_{G9}$ and $rACT_{G9}$, bound to hK14 with association constants of 217 000 and 74 000 $M^{-1}\cdot s^{-1}$, respectively, while $rACT_{E8}$ yielded higher association constant of 575 000 $M^{-1}\cdot s^{-1}$ (Table 2).

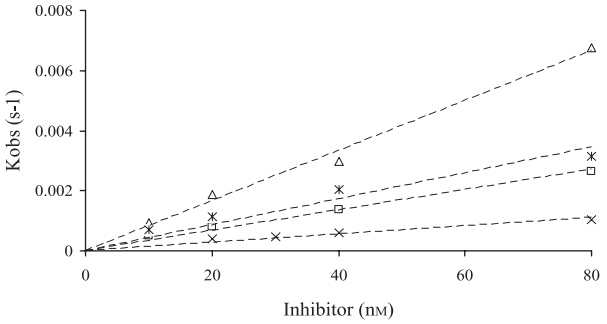

Fig. 4. Inhibition of hK14 by rAAT, rACT and their variants under first order conditions. hK14 (2 nM) and Boc-Val-Pro-Arg-AMC substrate (20 μM) were added to different concentrations (0–80 nM) of AATwt (*), AAT_{G9} (□), ACT_{E8} (△) and ACT_{G9} (x) for 45-min reactions at 37 °C.

Inhibitory specificity of recombinant rAAT and rACT variants

In order to define the inhibitory specificity of the developed hK14 inhibitors, we investigated the reaction of purified variants with a large panel of proteases. First, proteases with broad specificities were examined, including trypsin, chymotrypsin, plasma kallikrein, human neutrophil elastase and thrombin. Then, we assessed the specificity of hK14 inhibitors towards enzymes belonging to the same protease family, i.e. hK2, hK3, hK5, hK6, hK8 and hK13 (Table 3). After a 30-min incubation of hK14 with an excess of inhibitors ($[I]_0/[E]_0$ of 50 : 1), no residual activity could be detected with all modified serpins and rAATwt. Under these conditions, rACTwt showed a weak (17%) inhibition towards hK14. Serpins modified with the E8 substrate showed moderate specificity,

Table 3. Inhibitory specificity of hK14 inhibitors. Inhibition percentage corresponding to 100 *[1–(velocity in presence of inhibitor/velocity of uninhibited control)]. Reaction of 30 min incubation with an excess of inhibitor ($[I]_0/[E]_0$ of 50 : 1).

Protease	AATwt	AAT_{E8}	AAT_{G9}	ACTwt	ACT_{E8}	ACT_{G9}
hK14	100	100	100	17	100	100
Trypsin	100	100	0	0	100	0
Chtr	100	19	100	100	14	100
PK	17	100	0	46	36	0
HNE	100	0	0	16	0	0
Thrombin	4	0	0	18	0	0
hK2	0	19	0	0	100	0
hK3	0	0	0	100	0	0
hK5	28	100	30	7	100	0
hK6	33	100	0	24	72	0
hK8	0	36	0	0	34	0
hK13	0	30	0	0	0	0

since several other enzymes were inhibited by these inhibitors. However, very high specificity was observed with $rAAT_{G9}$ and $rACT_{G9}$, as none of the tested enzymes was inhibited, except chymotrypsin and to a lower extent hK5.

Discussion

The human tissue kallikrein family is a group of serine proteases that are expressed in diverse tissues and are thought to be involved in many physiological processes [21]. Coexpression and coordinated regulation of many of these proteases led to the hypothesis that they participate in enzymatic cascades. They could also be involved in diverse pathological processes, including ovarian and breast cancer progression, malignancies in which human kallikrein 14 is overexpressed [15]. Recently, we used phage display technology to study the substrate specificity of hK14, allowing the isolation of highly specific and sensitive substrate peptides. Furthermore, several potential human target proteins, which are involved in cancer biology, have been proposed as hK14 substrates [19].

To investigate the potential biological roles and therapeutic applications of hK14, new tools, such as specific inhibitors, are needed. We used information from the analysis of substrate peptide sequences obtained by phage display to develop specific inhibitors to hK14. Since hK14 has dual activity, trypsin and chymotrypsin-like [19], we opted to examine the serpins AAT and ACT. Their complementary protease inhibitory profile provided attractive backbones for the construction of novel hK14 inhibitors. It has been previously demonstrated that some kallikreins were recovered *in vivo* as complexes with natural serpins, such as PSA with ACT, AAT [22] and PCI [23], hK2 with PCI [24,25] and ACT [26], hK1 with kallistatin [27], and hK6 with ACT [28]. To date, no natural inhibitor of hK14 has been identified. Our results indicate that AAT and ACT could be two potential physiological inhibitors of hK14, with AAT demonstrating very high inhibition parameters. Like all members of the serpin superfamily, AAT and ACT are characterized by a dominant β-sheet A and a mobile reactive loop that acts as a pseudo-substrate for the target proteases [29,30]. Following the cleavage of the P1-P1′ bond within the RCL, a covalent acyl-enzyme bond between the inhibitor and the target enzyme is formed and the protease is trapped within an irreversible complex by insertion of the cleaved loop into the β-sheet A [31,32]. Thus, amino acids in this region of the RCL are closely related to the active site of the protease and affect binding, cleavage and covalent bond formation.

In this study, we used site-directed mutagenesis to develop AAT and ACT variants in which specific hK14 substrate peptides were introduced into the RCL. It has been previously shown that replacement of RCL residues can enhance the inhibitory properties of a serpin, as well as transform the modified inhibitor into a simple substrate for the target protease [33,34]. Several studies examined the effects of mutations within the RCL; changing residues near the scissile bond can lead to alterations of the stoichiometry of inhibition and the association rate constant with different target proteases [35–37].

In addition to the proven importance of P1, nearby residues could also play an essential role. In the case of AAT, even with the same P4-P4′ residues, changes in the RCL led to decrease of constant rates [38], whereas substitutions at P4′ and P5′ residues of the plasminogen activator inhibitor-1 (PAI-1) also resulted in considerable changes in the constant rates [39]. We therefore restricted the mutations to P4-P2′ residues as was previously done with hK2 inhibitor development [20].

Recombinant serpins were fused to a His-tag to facilitate purification of the soluble protein from *Escherichia coli*, avoiding any refolding protocol from inclusion bodies. Despite the presence of this N-terminal His-tag and the bacterial production system, the purified recombinant serpins exhibited high reactivity towards proteolytic enzymes. Indeed, the inhibition parameters, SI and rate constant of inhibition (k_a) of wild-type recombinant serpins were similar to those which are commercially available and have been purified from natural sources (data not shown).

Besides, modification of the RCL of the two newly generated AAT variants, did not induce any major kinetic effect (k_a and SI) compared to the wild-type serpin. However, ACT variants, clearly demonstrated a higher inhibitory activity towards hK14 than the wild-type serpin. Although rAATwt was more efficient than rACTwt as an hK14 inhibitor, there was no clear advantage to use this backbone for hK14 inhibitor construction since $rACT_{E8}$ showed a higher inhibition rate than both recombinant AATs. Moreover, complexes formed by AAT variants were less stable than those formed by ACT.

The specific structural differences between these two backbones that allow or disallow an efficient distortion of the active site of hK14 are not defined but such variation of complex breakdown rates has been previously reported regarding human neutrophil elastase [34]. This stability time is, however, expected to be long enough for a potential *in vivo* application of the inhibitor, as protease-serpin complexes are usually

cleared from tissues or plasma relatively rapidly [40–42].

Independent of the serpin scaffold, the variants obtained from modification with the G9 peptide demonstrated less inhibitory efficiency than variants with the E8 peptide. These results are in good agreement with kinetic analysis data of peptide substrates, which demonstrated that hK14 has a higher activity towards substrates with an Arg residue in position P1, such as peptide E8 [19].

The amino acid sequences that comprise the recognition motif in serpin variants were chosen according to the cleavage preference and the specificity of hK14 for its substrates. As expected, AAT and ACT serpins modified with the G9 sequence, which lacks an Arg residue, did not exert any inhibitory activity against proteases with trypsin-like specificity, in contrast to variants with Arg at the P1 position, which display a rather broad inhibitory spectrum towards other serine proteases. This corresponds to our previous observation that peptide G9 is highly specific to hK14 [19]. The marked specificity of hK14 for the cleavage site within the RCL might be important for a potential *in vivo* or therapeutic application of such an inhibitor, in order to avoid inactivation by other proteases, either by complex formation or by degradation.

This is the first report describing the development of highly specific inhibitors for hK14. Using two different backbones, we developed four recombinant inhibitors, two of which demonstrated high specificity. Preliminary studies on hK14 expression in various tumors support that this enzyme may be involved in cancer progression. The recombinant inhibitors might be useful in studies aiming to better understand the physiological and pathological roles of this kallikrein and for assessing their potential as therapeutic targets.

Experimental procedures

Materials

The following materials were obtained from commercial sources: elastase, trypsin, chymotrypsin, thrombin and plasma kallikrein (Calbiochem, Lucerne, Switzerland), T4 DNA ligase (Invitrogen, Basel, Switzerland), T4 polynucleotide kinase (Qbiogene, Basel, Switzerland), Ni^{2+}-nitrilotriacetic acid agarose beads (Qiagen, Basel, Switzerland), restrictions enzymes (Roche, Mannheim, Germany; Amersham Pharmacia, Piscataway, USA; Promega, Buchs, Switzerland), anti-His antibody and alkaline phosphatase-conjugated goat antimouse secondary antibody (Sigma, Buchs, Switzerland). Fluorescent substrates Z-Phe-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Gly-Gly-Arg-AMC and MeOSuc-Ala-Ala-Pro-Val-AMC were purchased from Calbiochem (Lucerne, Switzerland), Boc-Val-Pro-Arg-AMC from Bachem (Bubendorf, Switzerland), Abz-Thr-Phe-Arg-Ser-Ala-Dap(Dnp)-NH_2 from Neosystem (Strasbourg, France). Oligonucleotide synthesis was carried out by Invitrogen (Basel, Switzerland) and DNA sequencing by Synergene Biotech GmbH (Schlieren, Switzerland). Human kallikreins 2, 5, 13 and 14 were produced in the *Pichia pastoris* expression system, as previously described [11,19,43]. Human kallikrein 6 was produced in 293 human embryonic kidney cells and human kallikrein 8 with a baculovirus vector in HighFive (Invitrogen, Burlington, Canada) insect cells [44,45]. hK6 and hK8 were activated with lysyl-carboxypeptidase [46].

Construction of expression vectors for recombinant wild-type AAT (rAATwt), ACT (rACTwt) and their variants

Human AAT cDNA (Invitrogen) was amplified by PCR using the oligonucleotides 5′-TATGGATCCGATGATCCC CAGGGAGA-3′ and 5′-CGCGAAGCTTTTATTTTTGG GTGGGA-3′. The *Bam*HI-*Hind*III fragment of the amplified AAT gene was cloned into the vector pQE9 (Qiagen) resulting in plasmid pAAT, which contains the open reading frame of mature AAT with an N-terminal His_6-tag. Silent mutations producing *Kas*I and *Bsu*36I restriction sites were introduced in pAAT 24 bp upstream and 11 bp downstream of the P1 codon of the RCL domain, respectively. The restriction sites were created using the oligonucleotides 5′-ACTGAAGCTGCTGGCGCCGAGCTCTTAGAGGCC ATA-3′ for the *Kas*I site and 5′-GTCTATCCCCCCTGAG GTCAAGTTC-3′ for the *Bsu*36I site following the QuikChange mutagenesis protocol supplied by Stratagene. Construction of the plasmid expressing wild-type ACT was described previously [1].

Recombinant (r) rAAT and rACT variants were produced by replacement of the RCL region with corresponding DNA fragments amplified from appropriate template oligonucleotides: $rAAT_{E8}$, 5′ -CCATGTTTCTAGAGGCTCTGCAGC GTGCTATCCCGCCTGAGGTCAAGTT-3′; $rAAT_{G9}$, 5′-CCATGTTTCTAGAGACCGTTGACTACGCTATCCCG CCTGAGGTCAAGTT-3′, $rACT_{E8}$, 5′-TACCGCGGTCA AAATCCTGCAGCGTGCTATCCTGGTGGAGACGCG TGA-3′ and $rACT_{G9}$, 5′-TACCGCGGTCAAAACCGTTG ACTACGCTGCTCTGGTGGAGACGCGTGA-3′. Templates were amplified using primers corresponding to their respective flanking regions, 5′-GCTGGCGCCATGTTTCT AGAG-3′ and 5′-TTGTTGAACTTGACCTCAGG-3′ for AAT variants and 5′-GTACCGCGGTCAAA-3′ and 5′-TC ACGCGTGTCCAC-3′ for ACT variants. Resulting PCR fragments were cloned as *Kas*I/*Bsu*36I fragments into pAAT and as MluI/SacII fragments into rACTwt constructs and confirmed by DNA sequencing. Changes in the reactive site loop between positions P4 and P2′ are shown in Table 1.

Expression and purification of recombinant serpins

Recombinant serpins (wild type and variants) were produced in *E. coli* strain TG1. Cells were grown at 37 °C in 2x TY media (16 g tryptone, 10 g yeast extract, 5 g NaCl per L) containing 100 $\mu g \cdot mL^{-1}$ ampicillin to $OD_{600} = 0.5$–0.7. Isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 0.5 mM and 0.1 mM for production of rACT proteins and rAAT proteins, respectively. The recombinant serpins were expressed for an induction period of 16 h at 18 °C. Cells were harvested by centrifugation and resuspended in 0.1 volume of cold NaCl/P_i 2X. After 45 min of incubation with lysozyme (0.5 $mg \cdot mL^{-1}$) on ice, total soluble cytoplasmic proteins were extracted by four cycles of freeze/thaw and total DNA was degraded with DNase I. Cell debris was removed by centrifugation (25 min, 17 500 ***g***) and Ni^{2+}-nitrilotriacetic acid affinity agarose beads were added to the supernatant for 90 min at 4 °C to bind recombinant serpins. The resin was washed three times with 50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM imidazole and bound proteins were eluted with 50 mM Tris, pH 7.5, 150 mM NaCl, 150 mM imidazole. Eluted proteins were dialyzed against 50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Triton X-100 for 16 h at 4 °C and protein purity was assessed by Coomassie blue-stained SDS/PAGE. Protein concentrations were determined by the bicinchoninic acid method [47], using bovine serum albumin as standard (Pierce Chemical Co., Rockford, IL, USA).

Stoichiometry of inhibition (SI)

SI values of rAAT, rACT, and their variants were determined by incubating hK14 with varying concentrations of each inhibitor. After a 4 h incubation at 37 °C in reaction buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Triton X-100, 0.01% bovine serum albumin (BSA)], the residual hK14 activity was detected by addition of fluorescent substrate Boc-Val-Pro-Arg-AMC. Fluorescence was measured with excitation at 340 nm (± 15) and emission at 485 nm (± 10) in black 96 well plates using an FL_x800 fluorescence microplate reader (Bio-Tek Instruments, Inc., USA). The SI value corresponds to the abscissa intercept of the linear regression analysis of fractional velocity (velocity of inhibited enzyme reaction (v_i)/velocity of uninhibited enzyme reaction (v_0)) versus the molar ratio of the inhibitor to enzyme ($[I]_0/[E]_0$).

Kinetic analysis

The association rate constants for interactions of hK14 with different inhibitors were determined under pseudo-first order conditions using the progress curve method [48]. Under these conditions, a fixed amount of enzyme (2 nM) was mixed with different concentrations of inhibitor (0–80 nM) and an excess of substrate (20 μM). Reactions were performed in reaction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0,05% Triton X-100, 0.01% BSA) at 37 °C for 45min and the rate of product formation was measured using the FL_x800 fluorescence microplate reader. Inhibition is considered to be irreversible over the course of the reaction and the progression of enzyme activity is expressed as product formation (P), beginning at a rate (v_z) and is inhibited over time (t) at a first-order rate (k_{obs}), where the rate constant is only dependent on the inhibitor concentration.

$$P = (v_z/k_{obs}) \times [1 - e^{(-kobst)}] \quad (1)$$

A k_{obs} was calculated for five different concentrations of each inhibitor, by nonlinear regression of the data using Equation 1. By plotting k_{obs} versus inhibitor concentration [I], a second–order rate constant, k', equal to the slope of the curve ($k' = \Delta k_{obs}/\Delta[I]$), was determined. Due to the competition between the inhibitor and the substrate, Equation 2 is used to correct the second order rate constant k' by taking into account the substrate concentration [S] and the K_m of the enzyme for its substrate, giving the k_a.

$$k_a = (1 + [S]/K_m) \times k' \quad (2)$$

The K_m of hK14 for the substrate MeOSuc-Val-Pro-Arg-AMC was 8 μM.

SDS/PAGE analysis of enzyme–inhibitor complexes

A constant amount of each inhibitor (1 μg) was incubated for 4 h in reaction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0,05% Triton X-100) with varying amounts of hK14 leading to $[I]_0/[E]_0$ ratios of 0.6, 1.2, 2.4 (ACT_{E8}) and 0.75, 1.5 and 3 (ACT_{G9}). Samples were heated at 90 °C for 10 min, resolved on a 10% SDS gel under reducing conditions and visualized by Coomassie blue staining.

Inhibitory specificity of recombinant rAAT and rACT variants

Two nanomoles of trypsin, chymotrypsin, plasma kallikrein, human neutrophil elastase and thrombin and 10 nM of hK2, hK3, hK5, hK6, hK8, hK13 and hK14 were incubated for 30 min at 37 °C with 100 nM and 500 nM of recombinant inhibitors, respectively. Residual activities were detected by the addition of fluorescent substrates (Z-Phe-Arg-AMC for trypsin and plasma kallikrein, Suc-Ala-Ala-Pro-Phe-AMC for chymotrypsin, Z-Gly-Gly-Arg-AMC for thrombin, MeOSuc-Ala-Ala-Pro-Val-AMC for human neutrophil elastase and Abz-Thr-Phe-Arg-Ser-Ala-Dap(Dnp)-NH_2 for human kallikreins).

Stability of the complex

hK14 (2 nM) was incubated with ACT_{E8} ($[I]_o/[E]_o = 2.4$) and ACT_{G9} ($[I]_o/[E]_o = 3$) for 45min, 4, 8 and 24 h at 37 °C in reaction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Triton X-100, 0.01% BSA). The residual activity was then detected by addition of 20 μM of the fluorescent substrate Boc-Val-Pro-Arg-AMC. It was calculated as a percentage of uninhibited hK14 activity incubated under the same conditions. Uninhibited hK14 activity decreased to 98, 73, 67 and 30% after 45 min, 4, 8 and 24 h incubation at 37 °C, respectively.

Acknowledgements

This work is supported by CTI agency, OPO foundation and Med Discovery, Switzerland.

References

1 Diamandis EP, Yousef GM, Luo LY, Magklara A & Obiezu CV (2000) The new human kallikrein gene family: implications in carcinogenesis. *Trends Endocrinol Metab* **11**, 54–60.
2 Yousef GM & Diamandis EP (2001) The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocr Rev* **22**, 184–204.
3 Borgoño CA, Michael IP & Diamandis EP (2004) Human tissue kallikreins: physiologic roles and applications in cancer. *Mol Cancer Res* **2**, 257–280.
4 Clements JA, Willemsen NM, Myers SA & Dong Y (2004) The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers. *Crit Rev Clin Laboratory Sci* **41**, 265–312.
5 Tremblay RR, Deperthes D, Tetu B & Dube JY (1997) Immunohistochemical study suggesting a complementary role of kallikreins hK2 and hK3 (prostate-specific antigen) in the functional analysis of human prostate tumors. *Am J Pathol* **150**, 455–459.
6 Becker C, Piironen T, Pettersson K, Bjork T, Wojno KJ, Oesterling JE & Lilja H (2000) Discrimination of men with prostate cancer from those with benign disease by measurements of human glandular kallikrein 2 (HK2) in serum. *J Urol* **163**, 311–316.
7 Haese A, Graefen M, Steuber T, Becker C, Noldus J, Erbersdobler A, Huland E, Huland H & Lilja H (2003) Total and Gleason grade 4/5 cancer volumes are major contributors of human kallikrein 2, whereas free prostate specific antigen is largely contributed by benign gland volume in serum from patients with prostate cancer or benign prostatic biopsies. *J Urol* **170**, 2269–2273.
8 Anisowicz A, Sotiropoulou G, Stenman G, Mok SC & Sager R (1996) A novel protease homolog differentially expressed in breast and ovarian cancer. *Mol Med* **2**, 624–636.
9 Dhar S, Bhargava R, Yunes M, Li B, Goyal J, Naber SP, Waser DE & Band V (2001) Analysis of normal epithelial cell specific-1 (NES1)/kallikrein 10 mRNA expression by *in situ* hybridization, a novel marker for breast cancer. *Clin Cancer Res* **7**, 3393–3398.
10 Chang A, Yousef GM, Scorilas A, Grass L, Sismondi P, Ponzone R & Diamandis EP (2002) Human kallikrein gene 13 (KLK13) expression by quantitative RT-PCR: an independent indicator of favourable prognosis in breast cancer. *Clin Cancer Res* **86**, 1457–1464.
11 Yousef GM, Polymeris ME, Grass L, Soosaipillai A, Chan PC, Scorilas A, Borgono C, Harbeck N, Schmalfeldt B, Dorn J *et al.* (2003) Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. *Cancer Res* **63**, 3958–3965.
12 Luo LY, Yousef G & Diamandis EP (2003) Human tissue kallikreins and testicular cancer. *APMIS* **111**, 225–233.
13 Yousef GM, Magklara A, Chang A, Jung K, Katsaros D & Diamandis EP (2001) Cloning of a new member of the human kallikrein gene family, KLK14, which is down-regulated in different malignancies. *Cancer Res* **61**, 3425–3431.
14 Hooper JD, Bui LT, Rae FK, Harvey TJ, Myers SA, Ashworth LK & Clements JA (2001) Identification and characterization of KLK14, a novel kallikrein serine protease gene located on human chromosome 19q13.4 and expressed in prostate and skeletal muscle. *Genomics* **73**, 117–122.
15 Borgoño CA, Grass L, Soosaipillai A, Yousef GM, Petraki CD, Howarth DH, Fracchioli S, Katsaros D & Diamandis EP (2003) Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. *Cancer Res* **63**, 9032–9041.
16 Yousef GM, Fracchioli S, Scorilas A, Borgono CA, Iskander L, Puopolo M, Massobrio M, Diamandis EP & Katsaros D (2003) Steroid hormone regulation and prognostic value of the human kallikrein gene 14 in ovarian cancer. *Am J Clin Pathol* **119**, 346–355.
17 Yousef GM, Borgono CA, Scorilas A, Ponzone R, Biglia N, Iskander L, Polymeris ME, Roagna R, Sismondi P & Diamandis EP (2002) Quantitative analysis of human kallikrein gene 14 expression in breast tumours indicates association with poor prognosis. *Br J Cancer* **87**, 1287–1293.
18 Yousef GM, Stephan C, Scorilas A, Ellatif MA, Jung K, Kristiansen G, Jung M, Polymeris ME & Diamandis EP (2003) Differential expression of the human kallikrein gene 14 (KLK14) in normal and cancerous prostatic tissues. *Prostate* **56**, 287–292.
19 Felber LM, Borgoño CA, Cloutier SM, Kündig C, Kishi T, Chagas JR, Jichlinski P, Gygi CM, Leisinger

HJ, Diamandis EP *et al*. (2004) Enzymatic profiling of human kallikrein 14 using phage-display substrate technology. *Biol Chem* **386**, 291–298.
20 Cloutier SM, Kündig C, Felber LM, Fattah OM, Chagas JR, Gygi CM, Jichlinski P, Leisinger HJ & Deperthes D (2004) Development of recombinant inhibitors specific to human kallikrein 2 using phage-display selected substrates. *Eur J Biochem* **271**, 607–613.
21 Borgono CA & Diamandis EP (2004) The emerging roles of human tissue kallikreins in cancer. *Nat Rev Cancer* **4**, 876–890.
22 Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K & Alfthan O (1991) A complex between prostate-specific antigen and alpha 1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer. *Cancer Res* **51**, 222–226.
23 Espana F, Sanchez-Cuenca J, Vera CD, Estelles A & Gilabert J (1993) A quantitative ELISA for the measurement of complexes of prostate-specific antigen with protein C inhibitor when using a purified standard. *J Lab Clin Med* **122**, 711–719.
24 Deperthes D, Chapdelaine P, Tremblay RR, Brunet C, Berton J, Hebert J, Lazure C & Dube JY (1995) Isolation of prostatic kallikrein hK2, also known as hGK-1, in human seminal plasma. *Biochim Biophys Acta* **1245**, 311–316.
25 Deperthes D, Frenette G, Brillard-Bourdet M, Bourgeois L, Gauthier F, Tremblay RR & Dube JY (1996) Potential involvement of kallikrein hK2 in the hydrolysis of the human seminal vesicle proteins after ejaculation. *J Androl* **17**, 659–665.
26 Grauer LS, Finlay JA, Mikolajczyk SD, Pusateri KD & Wolfert RL (1998) Detection of human glandular kallikrein, hK2, as its precursor form and in complex with protease inhibitors in prostate carcinoma serum. *J Androl* **19**, 407–411.
27 Zhou GX, Chao L & Chao J (1992) Kallistatin: a novel human tissue kallikrein inhibitor. Purification, characterization, and reactive center sequence. *Biochem J* **339**, 473–479.
28 Hutchinson S, Luo LY, Yousef GM, Soosaipillai A & Diamandis EP (2003) Purification of human kallikrein 6 from biological fluids and identification of its complex with alpha (1)-antichymotrypsin. *Clin Chem* **49**, 746–751.
29 Baumann U, Huber R, Bode W, Grosse D, Lesjak M & Laurell CB (1991) Crystal structure of cleaved human alpha 1-antichymotrypsin at 2.7 Å resolution and its comparison with other serpins. *J Mol Biol* **218**, 595–606.
30 Wei A, Rubin H, Cooperman BS & Christianson DW (1994) Crystal structure of an uncleaved serpin reveals the conformation of an inhibitory reactive loop. *Nat Struct Biol* **1**, 251–258.
31 Stratikos E & Gettins PG (1998) Mapping the serpin-proteinase complex using single cysteine variants of alpha1-proteinase inhibitor Pittsburgh. *J Biol Chem* **273**, 15582–15589.
32 Wilczynska M, Fa M, Karolin J, Ohlsson PI, Johansson LB & Ny T (1997) Structural insights into serpin-protease complexes reveal the inhibitory mechanism of serpins. *Nat Struct Biol* **4**, 354–357.
33 Luke C, Schick C, Tsu C, Whisstock JC, Irving JA, Bromme D, Juliano L, Shi GP, Chapman HA & Silverman GA (2000) Simple modifications of the serpin reactive site loop convert SCCA2 into a cysteine proteinase inhibitor: a critical role for the P3′ proline in facilitating RSL cleavage. *Biochemistry* **39**, 7081–7091.
34 Plotnick MI, Samakur M, Wang ZM, Liu X, Rubin H, Schechter NM & Selwood T (2002) Heterogeneity in serpin-protease complexes as demonstrated by differences in the mechanism of complex breakdown. *Biochemistry* **41**, 334–342.
35 Djie MZ, Le Bonniec BF, Hopkins PC, Hipler K & Stone SR (1996) Role of the P2 residue in determining the specificity of serpins. *Biochemistry* **35**, 11461–11469.
36 Chen VC, Chao L & Chao J (2000) Roles of the P1, P2, and P3 residues in determining inhibitory specificity of kallistatin toward human tissue kallikrein. *Biol Chem* **275**, 38457–38466.
37 Dufour EK, Denault JB, Bissonnette L, Hopkins PC, Lavigne P & Leduc R (2001) The contribution of arginine residues within the P6–P1 region of alpha 1-antitrypsin to its reaction with furin. *J Biol Chem* **276**, 38971–38979.
38 McRae B, Nakajima K, Travis J & Powers JC (1980) Studies on reactivity of human leukocyte elastase, cathepsin G, and porcine pancreatic elastase toward peptides including sequences related to the reactive site of alpha 1-protease inhibitor (alpha 1-antitrypsin). *Biochemistry* **19**, 3973–3978.
39 Ibarra CA, Blouse GE, Christian TD & Shore JD (2004) The contribution of the exosite residues of plasminogen activator inhibitor-1 to proteinase inhibition. *J Biol Chem* **279**, 3643–3650.
40 Pizzo SV, Mast AE, Feldman SR & Salvesen G (1988) *In vivo* catabolism of alpha 1-antichymotrypsin is mediated by the Serpin receptor which binds alpha 1-proteinase inhibitor, antithrombin III and heparin cofactor II. *Biochim Biophys Acta* **967**, 158–162.
41 Mast AE, Enghild JJ, Pizzo SV & Salvesen G (1991) Analysis of the plasma elimination kinetics and conformational stabilities of native, proteinase-complexed, and reactive site cleaved serpins: comparison of alpha 1-proteinase inhibitor, alpha 1-antichymotrypsin, antithrombin III, alpha 2-antiplasmin, angiotensinogen, and ovalbumin. *Biochemistry* **30**, 1723–1730.

42 Enghild JJ, Valnickova Z, Thogersen IB & Pizzo SV (1994) Complexes between serpins and inactive proteinases are not thermodynamically stable but are recognized by serpin receptors. *J Biol Chem* **269**, 20159–20166.

43 Kapadia C, Chang A, Sotiropoulo G, Yousef GM, Grass L, Soosaipillai A, Xing X, Howarth DH & Diamandis EP (2003) Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. *Clin Chem* **49**, 77–86.

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

45 Kishi T, Grass L, Soosaipillai A, Shimizu-Okabe C & Diamandis EP (2003) Human kallikrein 8: immunoassay development and identification in tissue extracts and biological fluids. *Clin Chem* **49**, 87–96.

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

47 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ & Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76–85.

48 Morrison JF & Walsh CT (1988) The behavior and significance of slow-binding enzyme inhibitors. *Adv Enzymol Relat Areas Mol Biol* **61**, 201–301.