Inhibition of the cysteine proteinases cathepsins K and L by the serpin headpin (SERPINB13): A kinetic analysis

ARTICLE *in* ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS · FEBRUARY 2003

Impact Factor: 3.02 · DOI: 10.1016/S0003-9861(02)00635-5 · Source: PubMed

11 AUTHORS, INCLUDING:

Arumugam Jayakumar
University of Texas MD Anderson Cancer C…
51 PUBLICATIONS **2,149** CITATIONS
SEE PROFILE

Ya'an Kang
University of Texas MD Anderson Cancer C…
32 PUBLICATIONS **843** CITATIONS
SEE PROFILE

Stephen Pak
University of Pittsburgh
43 PUBLICATIONS **976** CITATIONS
SEE PROFILE

Ying Henderson
University of Texas MD Anderson Cancer C…
36 PUBLICATIONS **1,212** CITATIONS
SEE PROFILE

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Archives of Biochemistry and Biophysics 409 (2003) 367–374
ABB
www.elsevier.com/locate/yabbi

Inhibition of the cysteine proteinases cathepsins K and L by the serpin headpin (SERPINB13): a kinetic analysis☆

Arumugam Jayakumar,[a] Ya'an Kang,[a] Mitchell J. Frederick,[a] Stephen C. Pak,[b] Ying Henderson,[a] Paula R. Holton,[a] Kenji Mitsudo,[a] Gary A. Silverman,[b] Adel K. EL-Naggar,[c] Dieter Brömme,[d] and Gary L. Clayman[a,e,*]

[a] *Department of Head and Neck Surgery, M.D. Anderson Cancer Center, The University of Texas, 1515 Holcombe Blvd., Houston, TX 77030-4095, USA*
[b] *Department of Pediatrics, Harvard Medical School, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115-5737, USA*
[c] *Department of Pathology, M.D. Anderson Cancer Center, The University of Texas, 1515 Holcombe Blvd., Houston, TX 77030-4095, USA*
[d] *Department of Human Genetics, Mount Sinai School of Medicine, Fifth Avenue at 100th Street, New York, NY 10029-6574, USA*
[e] *Department of Cancer Biology, M.D. Anderson Cancer Center, The University of Texas, 1515 Holcombe Blvd., Houston, TX 77030-4095, USA*

Received 13 September 2002, and in revised form 23 October 2002

Abstract

Headpin (SERPINB13) is a novel member of the serine proteinase inhibitor (Serpin) gene family that was originally cloned from a keratinocyte cDNA library. Western blot analysis using a headpin-specific antiserum recognized a protein with the predicted M_r of 44 kDa in lysates derived from a transformed keratinocyte cell line known to express headpin mRNA. Similarity of the reactive-site loop (RSL) domain of headpin, notably at the P1–P1′ residues, with other serpins that inhibit cysteine and serine proteinases suggests that headpin may inhibit similar proteinases. This study demonstrates that recombinant headpin indeed inhibits cathepsins K and L, but not chymotrypsin, elastase, trypsin, subtilisin A, urokinase-type plasminogen activator, plasmin, or thrombin. The second-order rate constants (k_a) for the inhibitory reactions of rHeadpin with cathepsins K and L were $5.1 \pm 0.6 \times 10^4$ and $4.1 \pm 0.8 \times 10^4\ M^{-1}\ s^{-1}$, respectively. Headpin formed SDS-stable complexes with cathepsins K and L, a characteristic property of inhibitory serpins. Interactions of the RSL domain of headpin with cathepsins K and L were indicated by cleavage of headpin near the predicted P1–P1′ residues by these proteinases. These results demonstrate that the serpin headpin possesses specificity for inhibiting lysosomal cysteine proteinases.

Keywords: Serine proteinase inhibitor; Headpin; Insect cells; Cysteine proteinases; Proteinase inhibition

Serine proteinase inhibitors (serpins)[1] are members of a large and growing superfamily of structurally related proteins participating in the regulation of complex physiological processes [1,2]. With the possible exception of SERPINB5 (maspin), all of the human ov-serpins inhibit various cysteine or serine proteinases [3–6].

☆ This work was supported in part by National Institute of Dental Research Grant 1-P50-DE11906-01 (G.L.C.), National Institute of Health Independent Award 1R01DE13954-01 (G.L.C.), National Institute of Health First Investigator Award R29DE11689 (G.L.C.), Cancer Center Support Grant CA60374-00, the Michael A. O'Bannon Endowment of Cancer Research, the Betty Berry Cancer Research Fund, funds from the M.D. Anderson Cancer Center's Tobacco Research Program and the Texas Legislative Tobacco Settlement, and National Institutes of Health Grants CA87006 (G.A.S.) and CA86002 (G.A.S.).

* Corresponding author. Fax: 1-713-794-4662.
E-mail address: gclayman@mdanderson.org (G.L. Clayman).

[1] *Abbreviations used:* serpin, serine proteinase inhibitor; HNSCC, head and neck squamous carcinoma cells; MALDI-MS, matrix-associated laser desorption ionization mass spectroscopy; High Five, *Trichoplusia ni-5B1-4*; Sf9, *Spodoptera frugiperda*; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS, tris-buffered saline; DTT, dithiothreitol; P1, passage 1; RSL, reactive-site loop; HNE, human neutrophil elastase; uPA, urokinase-type plasminogen activator; $(Z\text{-PR})_2$-R110, $(Z\text{-Pro-Arg})_2$-R110; $(Z\text{-FR})_2$-R110, $(Z\text{-Phe-Arg})_2$-R110; Succ-AAPF-*p*NA, succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide; MeO-Succ-AAPV-*p*NA, methoxy-Succ-Ala-Ala-Pro-Val-*para*-nitroanilide; VLK-*p*NA, Val-Leu-Lys-*para*-nitroanilide; EGR-*p*NA, Glu-Gly-Arg-*para*-nitroanilide; SI, the stoichiometry of inhibition; mAb, monoclonal antibody; M_r, average molecular weight; E-64, [1-[*N*-[(L-*3-trans*-carboxy-oxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane]; pNA, *p*-nitroaniline.

0003-9861/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0003-9861(02)00635-5

Several members lack protease-inhibitory capability and instead have other physiological roles [7,8]. Many functionally distinct serpins have also been identified in monocotyledon and nonvascular plants [9], and some in viruses [10], but none in yeast or bacteria [2]. In humans, more than 35 serpin members are known to participate in extracellular and intracellular physiological processes, including blood coagulation, inflammation, cell migration, fibrinolysis, complement activation, remodeling of the extracellular matrix, hormone transport, apoptosis, cancer growth, and metastasis [1,2,11].

Inhibitory serpins are proteins of about 45 kDa that contain a highly exposed flexible reactive-site loop (RSL) located near the C-terminus of the molecule; this site mimics an ideal proteinase substrate [1,2]. The putative target proteinase initially forms a noncovalent Michaelis-like complex through interactions with amino acid residues of the serpin flanking the scissile bond (P1–P1′). Attack of the active-site serine on the scissile bond leads to a covalent oxy ester linkage between Ser-195 of the serine proteinase and the backbone carbonyl of the serpin P1 residue, followed by cleavage of the RSL P1–P1′ scissile bond [2]. Inhibition of cysteine proteinases by serpins is postulated to involve an analogous mechanism, with the difference being that the kinetically trapped intermediate is actually a thiol ester involving the Cys-124 active site of the cysteine proteinase [2]. The detection of an SDS-stable complex between serpins and their target proteinases provides evidence for the formation of a stable, covalent linkage responsible for irreversible inhibition [2].

In previous studies, we identified, cloned, and mapped a novel serpin named headpin to chromosome 18q21.3 [12,13]. The cloning of hurpin/P113, which has a sequence identical to that of headpin, was also reported [14]. Although the target enzymes that interact with headpin/hurpin have not yet been identified, it has been suggested that headpin belongs to the inhibitory class of serpins [13,14]. In this manuscript, this hypothesis was confirmed by the finding that recombinant headpin (rHeadpin) is an inhibitor of lysosomal cysteine proteinases cathepsins K and L [5,15], but not chymotrypsin, elastase, trypsin, subtilisin A, uPA, plasmin, or thrombin. Overall, these results demonstrate that the serpin headpin possesses specificity for inhibiting papain-like cysteine proteinases.

Materials and methods

Materials

The following reagents were obtained commercially as indicated: Fall Army worm cell line *Spodoptera frugiperda* (Sf9) (American Type Culture Collection, Manassas, VA); low-melting-point agarose, CELLFECTIN, pFASTBAC HTb, *Escherichia coli* competent DH10BAC, cabbage looper egg cell line, *Trichoplusia ni-5B1-4* (High Five), and ultimate serum-free insect medium (Invitrogen, Carlsbad, CA); restriction endonucleases (New England Biolabs, Beverly, MA); TALON Superflow (Clontech Laboratory, Palo Alto, CA); insect-XPRESS and fetal bovine serum (BioWhittaker, Walkersville, MD); YM10 Centriplus (Millipore, Bedford, MA); precast SDS–PAGE gels and prestained markers (Bio-Rad, Hercules, CA); methanol (EM Science, Gibbstown, NJ); bovine serum albumin (Kabi Pharmacia, Franklin, OH); bromophenol blue (MCB Manufacturing Chemists, Cincinnati, OH); dithiothreitol and glycerol (Roche Applied Science, Indianapolis, IN).

Construction, transfection, and isolation of recombinant headpin baculovirus

The rHeadpin composite bacmid DNA was generated by subcloning a *Bam*HI/*Hin*dIII fragment containing the complete coding sequence of human headpin into the donor vector pFASTBAC HTb. The headpin cDNA is thus placed under the transcriptional regulation of the viral polyhedrin gene promoter. Recombinant headpin composite bacmids were generated and transfected into Sf9 cells using CELLFECTIN. A high-titer P3 virus was generated by two rounds of P1 amplification [16]. The headpin composite virus encodes a recombinant headpin fusion protein of 425 amino acids long, containing 391 amino acids derived from native headpin. The remaining 34 amino acids, which include a six-histidine sequence, were derived from vector sequences and are joined to headpin as an N-terminal fusion. The expected M_r of this protein is 48.2 kDa.

Production of recombinant proteins in insect cells

Large-scale production of human rHeadpin was done by infecting spinner cultures of High Five cells with recombinant baculovirus at a multiplicity of infection of 5, in ultimate serum-free insect medium. Four days after infection, the cell pellet was harvested and rHeadpin was purified from the cell lysate using TALON affinity chromatography followed by gel-filtration chromatography [17,18]. Typically, the cell pellet was resuspended at a ratio of $1.0–1.5 \times 10^7$ cells/ml in a lysis buffer containing 10 mM Tris–HCl (pH 8.0), 130 mM NaCl, 10 mM NaF, 10 mM sodium phosphate, 10 mM sodium pyrophospate, 1.0% (v/v) Triton X-100, 1.0 μg/ml each of leupeptin, pepstatin, aprotinin, and 5.0 μg/ml Pefabloc SC plus. The suspension was kept on ice for 1 h. The lysed cell suspension was sonicated (using 15-s pulses at 80 W for three times) and the cell debris was removed by

centrifugation. The cell-free extract was diluted with 2× binding buffer containing 40 mM Tris–HCl (pH 8.0), 260 mM NaCl, 2.0% (v/v) Triton X-100, and 10% (v/v) glycerol and loaded onto a 1.0 × 5-cm column containing 0.6 ml of TALON Superflow equilibrated in binding buffer. The column was washed with binding buffer until the A_{280} was less than 0.005. The protein was then step eluted with 150 mM imidazole in binding buffer. The fractions (eluates 1–10) containing headpin proteins were pooled, concentrated using an YM10 Centriplus, and further purified by gel-filtration chromatography using a Superose 12 column (Amersham Pharmacia Biotech, Sweden) preequilibriated in binding buffer. The column was washed with binding buffer and proteins were eluted at a flow rate of 10 ml/h and fractions containing pure rHeadpin were pooled, concentrated, and finally stored in binding buffer containing 15% glycerol at −80 °C.

Enzymes, inhibitors, and substrates

Human neutrophil elastase (HNE), human chymotrypsin, human trypsin, human cathepsin G, human plasmin, and human cathepsin L were purchased from Athens Research & Technology (Athens, GA). Papain and Pefablock SC plus were purchased from Roche Applied Science (Indianapolis, IN). Thrombin, coagulation factor Xa, and subtilisin A were obtained from Calbiochem-Novabiochem (San Diego, CA). Leupeptin, pepstatin, aprotinin, E-64, uPA, Succ-AAPF-*p*NA, MeO-Succ-AAPV-*p*NA, and VLK-*p*NA were purchased from Sigma Chemical (St. Louis, MO). (Z-PR_2-R110) and (Z-FR_2-R110) were purchased from Molecular Probes (Eugene, OR).

Polyacrylamide gel electrophoresis and Western blotting

Rabbit polyclonal antisera were raised against headpin by immunization of a New Zealand White rabbit with synthetic peptides from the headpin sequence (His^{58}-Lys^{69} and Ser^{272}-Lys^{280}), which had no sequence similarity to other serpins. Protein concentrations were estimated by the methods of Bradford using bovine serum albumin (BSA) as a standard [19]. Lysates and proteins were boiled for 10 min, centrifuged, and applied on a Miniprotein II electrophoresis system (Bio-Rad) for SDS–PAGE using a 10% slab gel as described [17,18,20]. Electrophoretic transfer of proteins from the polyacrylamide gel to a nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH) was achieved using a Mini-Transblot electrophoretic cell (Bio-Rad), which was run at 25 V for 16 h. After transfer, the nitrocellulose membrane was incubated overnight at room temperature with 5% milk in TBS or 3% BSA in TBS (for penta-his antibody) to block free binding sites on the membrane. The blocked nitrocellulose membranes were incubated for 2 h at room temperature with headpin-specific rabbit antiserum (1:2000 dilution) or mouse mAb (1:2000 dilution, Qiagen, Valencia, CA) specific for the histidine tag. Blots were washed three times for 10 min with TBS-Tween buffer and two times for 15 min with TBS alone. Subsequently, blots were incubated for 1 h with either horseradish peroxidase-conjugated goat-anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA) or horseradish peroxidase-conjugated goat-anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories), respectively. After four additional washes, blots were treated with ECL substrate (Amersham Bioscience, Piscataway, NJ) and exposed to Kodak X-AR5 films (Eastman Kodak Company Scientific Imaging Systems, Rochester, NY) for 1–3 min.

Matrix-associated laser desorption ionization mass spectroscopy (MALDI-MS)

Human cathepsin K (1.0 μM) or human cathepsin L (1.0 μM) in acetate reaction buffer (20 μl) was mixed with rHeadpin (3 μM) for 20 min at 25 °C and frozen at −80 °C. The mixture components were desalted, concentrated, and separated by MALDI-MS at the Wistar Protein Microchemistry Facility (Philadelphia, PA).

Other methods

The concentrations of enzymes were determined by active-site titration as described previously [6]. Thermostability assays, cysteine and serine proteinase inhibition assays, pseudo-first-order reactions, and stoichiometry of inhibition (SI) assays were performed as previously reported [3]. SI values and second-order rate constants for rHeadpin with cathepsin K or cathepsin L were determined as described previously [21]. Following SDS–PAGE and trypsin digestion, internal amino acid sequence analysis of the purified recombinant protein was performed by the Baylor College of Medicine-Protein Chemistry Core Laboratory (Houston, TX).

Results

Detection of native headpin protein

We previously reported that headpin mRNA is abundantly expressed by the immortalized human keratinocyte cell line, HaCaT, but absent from the head and neck squamous cell carcinoma (HNSCC) line UMSCC1 [13]. Therefore, total cellular lysates from these two cell lines were analyzed by Western blotting for the presence or absence of native headpin using a headpin-specific polyclonal rabbit antiserum. As shown

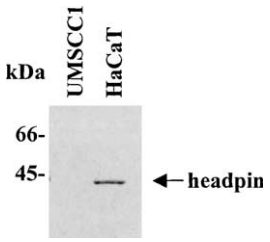

Fig. 1. Detection of native headpin in protein lysates. Total cellular lysates (75 μg) from either UMSCC1 or HaCaT cells were resolved by 10% SDS–PAGE, eletroblotting onto nitrocellulose, and detected with a headpin-specific antiserum (1:2000 dilution) raised against synthetic headpin peptides.

in Fig. 1, the headpin-specific antiserum recognized a single band of the expected molecular weight (M_r) of ~44 kDa in protein lysate from HaCaT, but not from UMSCC1. The results were consistent with the pattern of headpin mRNA expression previously reported for these two cell lines [13].

Purification and characterization of human rHeadpin

The results obtained from a typical purification procedure for rHeadpin are shown in Fig. 2. Enrichment of rHeadpin during various purification steps was demonstrated by SDS–PAGE (Fig. 2A) and Western blot analyses (Fig. 2B), respectively. A prominent band of ~48 kDa is stained by Coomassie blue in the lanes containing 150 mM imidazole eluates (Fig. 2A). The identity of TALON column-purified rHeadpin was confirmed by Western blotting and detection with an affinity-purified headpin-specific antiserum (Fig. 2B). Partial internal amino acid sequence analysis of this protein showed 100% identity with published cDNA-derived headpin protein sequences [12,14].

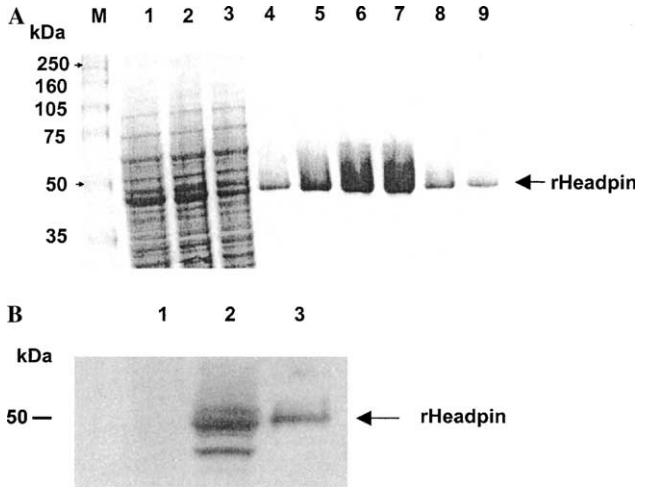

Fig. 2. SDS–PAGE and Western blot analyses of proteins at various stages of purification. (A) Fractions were analyzed by 10% SDS–PAGE, and the gel was stained with Coomassie brilliant blue R-250. Positions of molecular weight markers are noted to the left of the gel. The location of rHeadpin (arrow) is indicated. Lane 1, uninfected cell lysate (98 μg); lane 2, infected cell lysate (98 μg); lane 3, TALON flowthrough of infected cell lysate (90 μg); lane 4, eluate 1 (1.0 μg); lane 5, eluate 2, (2.0 μg); lane 6, eluate 3 (3.0 μg); lane 7, eluate 4 (4.0 μg); lane 8, eluate 5 (1.0 μg); lane 9, eluate 6 (1.0 μg). (B) Western blot analysis of various fractions using headpin polyclonal antibody. Lane 1, uninfected cell lysate (98 μg); lane 2, infected cell lysate (98 μg); lane 3, pooled eluates 1–6 (0.2 μg).

Survey of rHeadpin inhibitory activity

Comparison of the hinge region and putative scissile bond (P1–P1′) of headpin with other serpins suggested that headpin may function as an inhibitory serpin [2,12]. We therefore screened for serpin inhibitory activity by incubating rHeadpin with a panel of serine and cysteine proteinases prior to addition of the appropriate chromogenic or fluorogenic enzyme substrates (Table 1). Recombinant headpin inhibited completely the enzy-

Table 1
Inhibitory profile of rHeadpin

Proteinase (final concentration) (nM)	rHeadpin (final concentration) (nM)	Ratio (I/E)	Inhibition (%)[a]	Substrate (final concentration)
Cathepsin K (20)	200	10	99	Z-$(FR)_2$-R110 (5 μM)
Cathepsin L (25)	250	10	99	Z-$(FR)_2$-R110 (5 μM)
Chymotrypsin (50)	500	10	0	Succ-AAPF-pNA (1 mM)
HNE (50)	500	10	0	MeO-Succ-APPV-pNA (1 mM)
Human trypsin (10)	100	10	0	EGR-pNA (0.5 mM)
Subtilisin A (50)	500	10	0	Succ-AAPF-pNA (1 mM)
uPA (50)	500	10	0	EGR-pNA (0.5 mM)
Plasmin (50)	500	10	0	VLK-pNA (1 mM)
Thrombin (50)[b]	500	10	0	$(Z\text{-}PR)_2$-R110 (50 μM)
Cathepsin G (10)	100	10	50	Succ-AAPF-pNA (1 mM)
Papain (10)	100	10	40	Z-$(FR)_2$-R110 (5 μM)

[a] Proteinases and rHeadpin were incubated for 20 min at 25 °C. Residual enzyme activity was measured by adding substrate and measuring its hydrolysis over time [5]. Percent inhibition = 100 × [1 − (velocity in the presence of inhibitor/velocity of uninhibited control)].

[b] Thrombin inhibition was tested also in the presence of 0.1 and 5 μg/ml of heparin.

matic activity of cysteine proteinases cathepsins K and L, but not serine proteinases: chymotrypsin, elastase, trypsin, subtilisin A, uPA, plasmin, or thrombin. Recombinant headpin also showed modest inhibitory activity against cathepsin G and papain. However, subsequent SDS–PAGE analysis showed that this inhibition was due to a competition reaction, with the serpin serving as a simple substrate (not shown).

Kinetic analysis of the interaction between rHeadpin and target proteinases

The SI values for the interaction between rHeadpin and cathepsin K, and between rHeadpin and cathepsin L, were determined by incubating constant amounts of enzyme with different concentrations of headpin and measuring the residual enzymatic activity. The SI values were calculated by plotting the residual enzymatic activities versus the molar ratio of serpin to inhibitor. The SI values of rHeadpin with cathepsins K and L were approximately 1.4:1 and 1.9:1, respectively (Fig. 3). To determine the rate of complex formation between rHeadpin and each of these cathepsins, we performed a kinetic analysis under pseudo-first-order conditions using the progress curve method [6]. Each cathepsin was incubated with an excess of substrate and various concentrations of rHeadpin. The progress of enzyme inhibition was monitored over time (Figs. 4A and B, inset) and inhibition of initial reaction velocity represented as a simple decay, with a rate of k_{obs}. The k_{obs} values were plotted against the rHeadpin concentrations (Figs. 4A and B), and the slope of this line (k) and the K_m of the substrates were used to calculate the overall second-order rate constants. Using the mean value of three experiments, the k_a values for rHeadpin–cathepsin K and rHeadpin–cathepsin L were calculated to be $5.1 \pm 0.6 \times 10^4$ and $4.1 \pm 0.8 \times 10^4\,M^{-1}\,s^{-1}$, respectively.

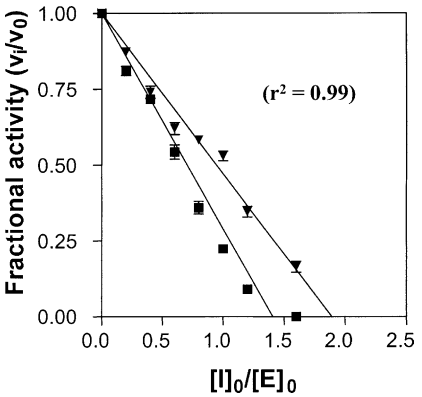

Fig. 3. SI of rHeadpin and the proteinases cathepsins K and L. Different concentrations of rHeadpin were incubated with 20 nM Cathepsin K (■) or 25 nM cathepsin L (▼) at 25 °C for 30 min and the residual cathepsin K or cathepsin L activity was measured. Fractional activity is the ratio of the velocity of inhibited enzyme (v_i) to the velocity of uninhibited control (v_o). Depicted results are averages of three independent experiments. In some instances, the error bars are not visible because of the symbols.

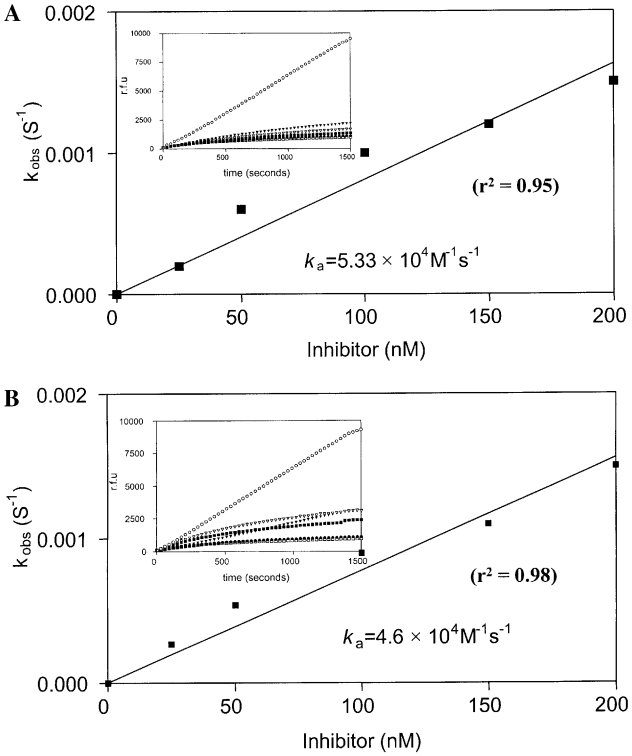

Fig. 4. Inhibition of cathepsins K and L by rHeadpin under pseudo-first-order conditions. Cathepsin K (A, inset) or cathepsin L (B, inset) progress curves with rHeadpin at 0 nM (○), 25 nM (▼), 50 nM (▽), 100 nM (■), 150 nM (●) 200 nM, (□). The first-order rate constant (k_{obs}) was calculated by a nonlinear regression fit of each curve. The first-order rate constant (k_{obs}) was plotted as a function of rHeadpin concentration for cathepsin K (A) or cathepsin L (B). The slope of this line (k) and the K_m of the substrates were used to calculate the overall second-order rate constants. In the above experiment k_a is for headpin–cathepsin K and headpin–cathepsin L were 5.33×10^4 and $4.60 \times 10^4\,M^{-1}\,s^{-1}$, respectively. The mean value of three experiments is reported under Results.

Analysis of complex formation of rHeadpin with proteinases

Most serpins form covalent complexes with their target proteinases. These complexes are resistant to denaturation by SDS, reducing agents, and heat [2]. To determine whether rHeadpin (48 kDa) forms a covalent complex with either cathepsin K (29 kDa), cathepsin L (26 kDa), papain (23 kDa), or cathepsin G (23 kDa), the serpin was mixed with each of these proteinases and samples were analyzed by SDS–PAGE and Western blotting. A higher M_r complex (~73 kDa) was apparent by SDS–PAGE only in reactions where rHeadpin was mixed with either cathepsin K or cathepsin L (data not shown); whereas, both papain and cathepsin G enzymes cleaved headpin into several smaller fragments (10–20 kDa). The presence of headpin–cathepsin K and

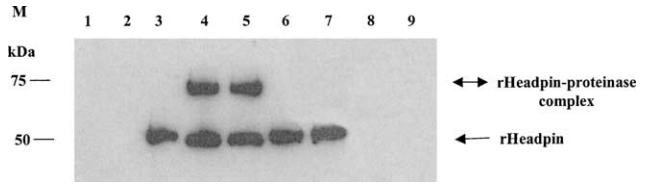

Fig. 5. Recombinant headpin–proteinase complexes. Enzyme, rHeadpin, or rHeadpin plus enzyme were incubated at 25 °C for 5 min. Gel loading buffer (2×) was added, each sample was heated to 95 °C for 5 min, and the proteins were separated by SDS–PAGE. Proteins were transferred to nitrocellulose and immunoblotted using headpin-specific antiserum (1:2000 dilution). Positions of molecular weight standards are noted to the left of the blot. Lane 1, cathepsin K alone (0.5 μg); lane 2, cathepsin L alone (0.5 μg); lane 3, purified rHeadpin alone (2.5 μg); lane 4, mixture of rHeadpin (2.5 μg) plus cathepsin K (0.5 μg); lane 5, mixture of rHeadpin (2.5 μg) plus cathepsin L (0.5 μg); lane 6, mixture of rHeadpin (2.5 μg) plus cathepsin K (0.5 μg) in which the latter was preincubated with 300 μM E-64; lane 7, mixture of rHeadpin (2.5 μg) plus cathepsin L (0.5 μg) in which the latter was preincubated with 300 μM E-64; lane 8, mixture of rHeadpin (2.5 μg) plus papain (0.5 μg); lane 9, mixture of rHeadpin (2.5 μg) plus cathepsin G (1.0 μg). The locations of the rHeadpin (arrow) and rHeadpin–proteinase complexes are indicated (double arrowheads). The molecular weights of rHeadpin, papain, cathepsin G, cathepsin K, and cathepsin L are 48,261, ~24,500, ~23,500, ~29,000, and ~24,000, respectively.

headpin–cathepsin L complexes was confirmed by Western blotting. A headpin-specific polyclonal antiserum detected headpin in the high-molecular-weight bands present only when rHeadpin was mixed with either cathepsin K or cathepsin L. (Fig. 5, lanes 4 and 5). As expected, no headpin band was visible in control lanes containing cathepsin K or cathepsin L, alone. Similarly, headpin was not detected in lanes where rHeadpin was mixed with either papain or cathepsin G (Fig. 5, lanes 8 and 9), suggesting efficient degradation of rHeadpin by these latter two enzymes. Formation of the high-M_r complexes was completely inhibited by preincubating cathepsins K and L with the active-site cysteine proteinase inhibitor E-64 (Fig. 5, lanes 6 and 7), consistent with a suicide inhibitory mechanism. These results suggest that the active site of cathepsins K and L is required for complex formation with rHeadpin and that rHeadpin forms tight complexes with cysteine proteinases in a manner similar to that observed for other serpins and their target serine proteinases [3]. Collectively, these data show that rHeadpin inhibited papain and cathepsin G by simple competition with the substrate, whereas rHeadpin inhibited cathepsins K and L by formation of a tight complex.

The reactive center of headpin

Because of the nature of the serpin inhibitory mechanism, location of the RSL cleavage site (P1–P1′) usually occurs ~17 residues downstream of the highly conserved *Glu* residue located in the proximal hinge region [2,22]. To locate its RSL cleavage site, rHeadpin

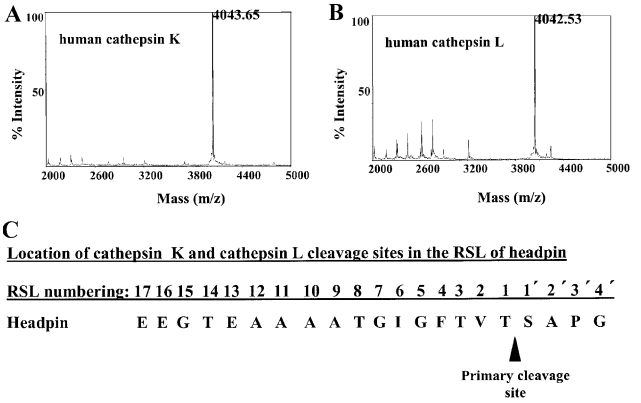

Fig. 6. Reactive center of headpin. Recombinant headpin (2–3 μg) was mixed with 0.9 μg of either cathepsin K (A) or cathepsin L (B) and incubated for 10 min at 25 °C. The reaction mixture was then analyzed by MALDI-MS. (C) The RSL of headpin showing the location of the primary cleavage site (triangle) based on the MALDI-MS data.

was incubated with either cathepsin K or cathepsin L for 5 min at 25 °C, and the resulting C-terminal cleavage fragments were resolved by MALDI-MS. For each proteinase and rHeadpin mixture, a major peak was detected at M_r 4.043 kDa (Fig. 6). These data confirm that the Thr-Ser at the putative P1–P1′ site is indeed the RSL cleavage site.

Discussion

Recently, we cloned the headpin cDNA from normal oral keratinocytes. Based on this cDNA sequence, headpin encodes a putative protein of 391 amino acids with significant homology to other serpin family members (39–59% identities) [2]. Western blotting with an antiserum specific for headpin-derived peptides has confirmed the existence and expression of a headpin protein with an apparent M_r of 44 kDa in an immortalized keratinocyte cell line. Based on a comparison of the consensus sequence for the RSL hinge region of headpin with other known serpins (e.g., SCCA1 and SCCA2) [2], it was predicted that headpin would be a functional inhibitory serpin. This prediction proved to be correct as our study establishes cathepsins K and L as key targets for inhibition by headpin.

Previous studies have used insect cell/baculovirus expression systems to produce functional serpins, among them maspin [23], human monocyte/neutrophil elastase inhibitor (HEI) [24], and neuroserpin [25]. The baculo-produced rHeadpin in the present work specifically inhibited cathepsins K and L, with a serpin-like mechanism. Theoretically serpins form tight complexes with their target proteinases at a stoichiometry of 1:1 when there is no deviation toward the substrate inhibitory pathway [2]. However, parallel substrate reactions can occur and this results in cleavage and inactivation of

serpin. A SI value greater than 1 is indicative of the degree to which serpin–proteinase complexes partition down this substrate pathway. For many inhibitory serpins, the measured SI values range from 1:1 to 2.5:1. [2,3,5,6,21,22]. The SI values for rHeadpin–cathepsin K and rHeadpin–cathepsin L interaction are well within this range.

The ability of rHeadpin to form SDS-stable complexes with its target proteinases confirmed that the inhibitory mechanism conforms to the suicide substrate-like inhibitory pathway described for most functional serpins [21]. Consistent with this suicide substrate-like inhibitory mechanism, cleavage of the RSL at the P1–P1′ bond by cathepsin K or cathepsin L releases a C-terminal ~4 kDa fragment. The observed MALDI-MS data for the C-terminal fragments released by cathepsins K and L, respectively, have a M_r of 4.04365 and 4.04253 kDa. This suggests that the Thr-Ser residues at the P1–P1′ position serve as cleavage sites both for cathepsins K and L, as predicted by amino acid sequence alignments with other serpins [2].

SCCA1 is a well studied serpin that inhibits cathepsins K, L, and S at 1:1 stoichiometry and with second-order rate constants $\geqslant 1 \times 10^5\,M^{-1}\,s^{-1}$ [5]. Using second-order rate constants (k_a) as a measure of potency, SCCA1 appears to be a better inhibitor of cathepsins K and L than headpin ($k_a \sim 0.5 \times 10^5\,M^{-1}\,s^{-1}$). However, a ranking based on k_a cannot in itself be used to predict the physiologic potency of these serpins in vivo. Various factors such as local concentration of the inhibitor, the presence of serpin inactivators, and the availability of serpin–proteinase complex clearance mechanisms [3] will affect the overall ability of a serpin to modulate activity of its target proteinase. Thus, a better understanding of the pattern of headpin expression, cathepsin K and L expression, secretion, and their local concentration in normal and abnormal conditions should help determine whether headpin is a physiologic inhibitor of cathepsins K and L.

Our previous observation that headpin mRNA expression is diminished in some oral cancer cell lines and oral SCC biopsy specimens [12,13] is interesting in light of our current finding that two headpin targets are cathepsins K and L proteinases. Cathepsin K has been identified as the major proteinase degrading collagen type I found in bone extracellular matrix [26,27]. One hallmark of the most aggressive oral malignancies is their potential to invade bone. Cathepsin L degrades collagen, laminin, elastin, and other protein components of basement membranes and recent studies have demonstrated that cathepsin L protein levels are significantly higher in the cytosol of tumor tissues of patients suffering from head and neck carcinoma [28,29]. Overexpression of cathepsin L has been linked to aggressive behavior in numerous tumors [30–34], including oral cancer [29]. The loss of headpin mRNA expression in both HNSCC cell lines and tumor biopsy specimens [12,13] could be advantageous for tumor cells that express the target proteinases cathepsins K and L. Other serpins such as maspin and PAI2 have also been shown to be down regulated in cancer [35,36]. Additional studies are required to characterize the expression levels of cathepsin K and headpin in normal, tumor, and surgical specimens of oral carcinoma.

Acknowledgments

We thank Walter Pagel of the M.D. Anderson Cancer Center, Department of Scientific Publications for critical scientific editing of the manuscript. We thank Drs. Christopher Tsu and Cliff Luke for their technical assistance and discussion, and Mary Wang and Katrina Briggs for managing the cell lines.

References

[1] J. Potempa, E. Korzus, J. Travis, J. Biol. Chem. 269 (1994) 15957–15960.
[2] G.A. Silverman, P.I. Bird, R.W. Carrell, F.C. Church, P.B. Coughlin, P.G. Gettins, J.A. Irving, D.A. Lomas, C.J. Luke, R.W. Moyer, J. Biol. Chem. 276 (2001) 33293–33296.
[3] C. Schick, Y. Kamachi, A.J. Bartuski, S. Cataltepe, N.M. Schechter, P.A. Pemberton, G.A. Silverman, J. Biol. Chem. 272 (1997) 1849–1855.
[4] J.K. Frazer, D.G. Jackson, J.P. Gaillard, M. Lutter, Y.J. Liu, J. Banchereau, J.D. Capra, V. Pascual, Eur. J. Immunol. 30 (2000) 3039–3048.
[5] C. Schick, P.A. Pemberton, G.P. Shi, Y. Kamachi, S. Cataltepe, A.J. Bartuski, E.R. Gornstein, D. Bromme, H.A. Chapman, G.A. Silverman, Biochemistry 37 (1998) 5258–5266.
[6] C. Luke, C. Schick, C. Tsu, J.C. Whisstock, J.A. Irving, D. Bromme, L. Juliano, G.P. Shi, H.A. Chapman, G.A. Silverman, Biochemistry 39 (2000) 7081–7091.
[7] D.W. Dawson, O.V. Volpert, P. Gillis, S.E. Crawford, H. Xu, W. Benedict, N.P. Bouck, Science 285 (1999) 245–248.
[8] Z. Liu, X. Zhou, S.D. Shapiro, J.M. Shipley, S.S. Twining, L.A. Diaz, R.M. Senior, Z. Werb, Cell 102 (2000) 647–655.
[9] C.E. Carter, H. Marriage, P.W. Goodenough, Biochemistry 39 (2000) 11005–11013.
[10] S.J. Turner, J. Silke, B. Kenshole, J. Ruby, J. Gen. Virol. 81 (2000) 2425–2430.
[11] S. Sheng, B. Truong, D. Fredrickson, R. Wu, A.B. Pardee, R. Sager, Proc. Natl. Acad. Sci. USA 95 (1998) 499–504.
[12] P. Spring, T. Nakashima, M. Frederick, Y. Henderson, G. Clayman, Biochem. Biophys. Res. Commun. 264 (1999) 299–304.
[13] T. Nakashima, S.C. Pak, G.A. Silverman, P.M. Spring, M.J. Frederick, G.L. Clayman, Biochim. Biophys. Acta 1492 (2000) 441–446.
[14] H.F. Abts, T. Welss, A. Mirmohammadsadegh, K. Kohrer, G. Michel, T. Ruzicka, J. Mol. Biol. 293 (1999) 29–39.
[15] N. Katunuma, A. Matsui, T. Kakegawa, E. Murata, T. Asao, Y. Ohba, Adv. Enzyme Regul. 39 (1999) 247–260.
[16] D.R. O'Reilly, L.K. Milleri, V.A. Luckow, Baculovirus Expression Vectors: A Laboratory Manual, Oxford University Press, Oxford, 1994.
[17] A. Jayakumar, W.Y. Huang, B. Raetz, S.S. Chirala, S.J. Wakil, Proc. Natl. Acad. Sci. USA 93 (1996) 14509–14514.

[18] A. Jayakumar, M.H. Tai, W.Y. Huang, W. al Feel, M. Hsu, L. Abu-Elheiga, S.S. Chirala, S.J. Wakil, Proc. Natl. Acad. Sci. USA 92 (1995) 8695–8699.
[19] A. Jayakumar, S.S. Chirala, S.J. Wakil, Proc. Natl. Acad. Sci. USA 94 (1997) 12326–12330.
[20] S.S. Chirala, W.Y. Huang, A. Jayakumar, K. Sakai, S.J. Wakil, Proc. Natl. Acad. Sci. USA 94 (1997) 5588–5593.
[21] M. Al Khunaizi, C.J. Luke, Y.S. Askew, S.C. Pak, D.J. Askew, S. Cataltepe, D. Miller, D.R. Mills, C. Tsu, D. Bromme, Biochemistry 41 (2002) 3189–3199.
[22] Y.S. Askew, S.C. Pak, C.J. Luke, D.J. Askew, S. Cataltepe, D.R. Mills, H. Kato, J. Lehoczky, K. Dewar, B. Birren, J. Biol. Chem. 276 (2001) 49320–49330.
[23] S. Sheng, P.A. Pemberton, R. Sager, J. Biol. Chem. 269 (1994) 30988–30993.
[24] T. Sugimori, J. Cooley, J.R. Hoidal, E. Remold-O'Donnell, Am. J. Respir. Cell Mol. Biol. 13 (1995) 314–322.
[25] G.A. Hastings, T.A. Coleman, C.C. Haudenschild, S. Stefansson, E.P. Smith, R. Barthlow, S. Cherry, M. Sandkvist, D.A. Lawrence, J. Biol. Chem. 272 (1997) 33062–33067.
[26] F.H. Drake, R.A. Dodds, I.E. James, J.R. Connor, C. Debouck, S. Richardson, E. Lee-Rykaczewski, L. Coleman, D. Rieman, R. Barthlow, J. Biol. Chem. 271 (1996) 12511–12516.
[27] W.S. Hou, Z. Li, R.E. Gordon, K. Chan, M.J. Klein, R. Levy, M. Keysser, G. Keyszer, D. Bromme, Am. J. Pathol. 159 (2001) 2167–2177.
[28] N. Vigneswaran, W. Zhao, A. Dassanayake, S. Muller, D.M. Miller, W. Zacharias, Hum. Pathol. 31 (2000) 931–937.
[29] P. Strojan, M. Budihna, L. Smid, B. Svetic, I. Vrhovec, J. Kos, J. Skrk, Clin. Cancer Res. 6 (2000) 1052–1062.
[30] S.S. Chauhan, L.J. Goldstein, M.M. Gottesman, Cancer Res. 51 (1991) 1478–1481.
[31] A. Dohchin, J.I. Suzuki, H. Seki, M. Masutani, H. Shiroto, Y. Kawakami, Cancer 89 (2000) 482–487.
[32] M. Sivaparvathi, M. Yamamoto, G.L. Nicolson, Z.L. Gokaslan, G.N. Fuller, L.A. Liotta, R. Sawaya, J.S. Rao, Clin. Exp. Metastasis 14 (1996) 27–34.
[33] L. Herszenyi, M. Plebani, P. Carraro, M. De Paoli, G. Roveroni, R. Cardin, Z. Tulassay, R. Naccarato, F. Farinati, Cancer 86 (1999) 1135–1142.
[34] N. Harbeck, U. Alt, U. Berger, R. Kates, A. Kruger, C. Thomssen, F. Janicke, H. Graeff, M. Schmitt, Int. J. Biol. Markers 15 (2000) 79–83.
[35] W. Xia, Y.K. Lau, M.C. Hu, L. Li, D.A. Johnston, S. Sheng, A. El Naggar, M.C. Hung, Oncogene 19 (2000) 2398–2403.
[36] C. Robert, I. Bolon, S. Gazzeri, S. Veyrenc, C. Brambilla, E. Brambilla, Clin. Cancer Res. 5 (1999) 2094–2102.