Biological activities of granzyme K are conserved in the mouse and account for residual Z-Lys-SBzl activity in granzyme A-deficient mice

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Abstract Tryptase-like activities of T and NK cells contribute to the induction of target cell apoptosis, but only granzyme A (GzmA) has been shown to exhibit Z-Lys-SBzl esterase activity in murine T cells. GzmA-deficient mice exhibit residual Z-Lys-SBzl hydrolyzing activity and almost normal levels of lymphocyte-mediated cytotoxicity. Here we report the cloning and biochemical characterization of recombinant mouse granzyme K (GzmK). The purified murine protein shows Z-Lys-SBzl hydrolyzing activity and is inhibited by bikunin, the light chain of inter-α-trypsin inhibitor, like the human homolog. We conclude that GzmK expressed by GzmA-deficient T cells accounts for the remaining Z-Lys-SBzl activity. Functional similarities between GzmA and GzmK may explain the subtle immunological deficits observed in GzmA-deficient mice.

Key words: Lymphocyte cytotoxicity; Bikunin; Granzyme; DNA fragmentation; Apoptosis; Inter-α-trypsin inhibitor

1. Introduction

Human granzyme K (granzyme 3, tryptase-2) has been purified from lymphokine-activated killer cells together with the granzymes A and B as a third lymphocyte specific granzyme [1] for which a murine homolog has not been inferred until recently [2]. Independently, a similar granzyme called RNK-tryptase-2 with hydrolytic activity for the thiobenzylesters Z-Lys-SBzl and Z-Arg-SBzl was isolated from the rat natural killer (NK) cell tumor RNK-16 and cloned at the cDNA level [3]. Rat granzyme K is identical with fragmentin-3 which was also characterized as an inducer of DNA fragmentation after perforin-mediated uptake into target cells [4]. The human homolog for granzyme K has recently been cloned at the cDNA level and shown to possess hydrolytic activities for the thioester compounds Z-Lys-SBzl and Z-Arg-SBzl [5–7]. In contrast, the murine homolog has only been identified at the genomic and cDNA level by virtue of its sequence homology to the rat and human sequences [2].

Granzyme A-deficient C57/Bl6 mice were first reported as being completely devoid of Z-Lys-SBzl and Z-Arg-SBzl cleaving activity in stimulated T cells and lymphoid organs [8]. Another granzyme A-deficient mouse strain obtained by homologous recombination on a 129/SvJ background, however, displayed significant levels of Z-Lys-SBzl cleaving activity in activated T cell populations [2]. This tryptase-like activity was detected after prolonged allogeneic stimulation in a mixed leukocyte reaction, but was not further characterized. In view of these discrepant data about a second Z-Lys-SBzl cleaving enzyme in murine T cells, we wished to analyze the functional properties of purified murine granzyme K in comparison with the recently characterized human granzyme K.

To this end, we have cloned and expressed the murine granzyme K cDNA in *Escherichia coli* and processed it to a functionally active enzyme as described [9]. Here we report that the murine granzyme K gene indeed encodes a Z-Lys-SBzl hydrolyzing enzyme which is rapidly inactivated by murine plasma. Like human granzyme K [9], the murine homolog interacts with the bikunin component of inter-α-trypsin inhibitor. Conservation of the tight binding between granzyme K and bikunin in men and mice strongly supports the biological relevance of this interaction.

2. Materials and methods

2.1. Isolation of the gene for mouse granzyme K

An almost full length cDNA probe for rat granzyme K was generated by PCR on the basis of published sequence data (GenBank accession number L19694) using the forward and backward primers DJ100 (5′-ATCTGCTCTGGTTTTCCTTGT-3′) and DJ131 (5′-AAACCACTTGTCATCTCAATGT-3′). This cDNA probe permitted us to identify the genomic clone C0164 in the murine P1 library ICRFp703 (strain C57/Bl6) (German resource center, Berlin). A 1.8 kb *Hin*dIII subfragment crossreacting with the rat cDNA was subcloned into the vector pUC18 and completely sequenced. This fragment contained exons 1 and 2 and part of intron 2. The exon 1 specific forward primer DJ301 (5′-TTTCTTCATGGGCTCTGGTTT-3′) was designed according to this genomic sequence and combined with a backward primer DJ39 (5′-AGGKCYYCCRGARTCHCC-3′) that anneals to the coding region of the highly conserved peptide GDSGGP containing the active site Ser-195 (chymotrypsin numbering).

2.2. Cloning of the cDNA of mouse GzmK and Northern blotting

PCR amplification of murine spleen cDNA (Clontech) with DJ301 and DJ39 yielded a 700 bp product that was also sequenced. A mouse GzmK specific forward primer DJ322 (5′-AAC AAG AAT GTC CAA CTG CTT-3′) located distal to DJ301 on this sequence was then designed to obtain the 3′ end of murine GzmK by 3′ RACE (rapid amplification of cDNA ends). The mGzmK specific forward primer DJ322 and the universal adaptor primer AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) were taken to generate a specific cDNA fragment from adaptor-ligated mouse spleen cDNA (Clontech) in a single PCR. The full length sequence of mouse GzmK was then assembled from these two cDNA segments. Northern blots were performed with total RNA from several cell lines including two Moloney leukemia virus-transformed T cell lymphomas, two thymoma lines (BW, L1210) and a CD4-positive (EL-4) and CD8-positive T cell line CTLL-2. After separation of RNA in formaldehyde agarose

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Abbreviations: GzmA, granzyme A; GzmK, granzyme K; Z-Lys-SBzl, N^{α}-benzyloxycarbonyl-L-lysine-thiobenzylester; PCR, polymerase chain reaction; Z, N^{α}-benzyloxycarbonyl; SBzl, thiobenzylester; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); CMK, chloromethyl ketone; TPCK, *N*-tosyl phenylalanine chloromethyl ketone; TLCK, *N*-tosyl lysine chloromethyl ketone

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gels, transfer onto nylon membranes and hybridization with a mouse cDNA probe, filter membranes were exposed to X-ray films for 2 days.

2.3. Recombinant expression in E. coli

The cDNA encoding the short N-terminal propeptide Met-Glu and the mature polypeptide chain of the mouse GzmK gene was amplified from mouse spleen cDNA using *Pfu*-DNA polymerase (Stratagene) and the gene specific forward and backward primers DJ601 (5′-TGTGTTTCCATATGGAAATTAT-3′) and DJ515 (5′-AATCTG-CAGTCTCAATGTGCA-3′), respectively. The resulting 750 bp PCR product was digested with the restriction enzymes *Nde*I and *Pst*I and subcloned into the vector pET24c(+) (Novagen). The cDNA sequence of this construct was confirmed by dideoxy sequencing and transformed into the *E. coli* strain B834(DE3) (Novagen) for protein expression. Inclusion body production, refolding, proteolytic conversion of mouse GzmK precursors by cathepsin C and purification was performed as described [9].

2.4. Activity assays

Hydrolytic activity of GzmK was assayed at room temperature (RT) in a buffer volume of 150 μl containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.01% Triton X-100, 0.3 mM DTNB (Sigma) and the respective thiobenzylester substrates at concentrations of 0.1 mM. Z-Lys-SBzl (Z, benzyloxycarbonyl; SBzl, thiobenzylester) (Sigma) was dissolved in water, Z-Arg-SBzl, Boc-Ala-Ala-Met-SBzl and Boc-Ala-Ala-Asp-SBzl (all from Enzyme Systems Products, Livermore, USA) in DMSO. Murine and human GzmK concentrations were determined by active site titration as described. Hydrolytic activity of 5 nM mouse GzmK was determined as the rate of hydrolysis by measuring increase of absorbance at 405 nm wavelength over 5 min using a Dynatech MR4000 microplate reader.

2.5. Inhibition studies

Various inhibitors (Sigma) were tested against 4 nM mouse GzmK using 0.3 mM Z-Lys-SBzl as the substrate. Equilibrium dissociation constants K_i were measured and calculated by non-linear regression analysis using 6 nM mGzmK, recombinant human bikunin [9] in the concentration range between 0 and 600 nM, and recombinant bikunin domain 2 [9] between 0 and 300 nM. Inhibition by plasma proteins was measured after adding 3 nM mGzmK to 40-fold diluted mouse plasma in the presence or absence of 0.5 U/ml heparin (Sigma). Residual enzyme activity was determined after 15 min of preincubation at RT as described above.

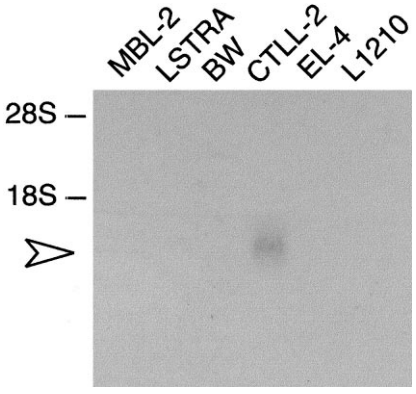

Fig. 1. Northern blot analysis of GzmK expression in different lymphoid cell lines. Total RNA from Moloney leukemia virus-transformed lymphoma lines (MBL-2, LSTRA), thymoma lines (BW, L1210) and CD4-positive (EL-4) and CD8-positive (CTLL-2) T cells was separated by formaldehyde/agarose gel electrophoresis, blotted onto a nylon membrane and probed with a radioactively labelled murine granzyme K cDNA probe. X-ray films were exposed for 2 days. On the left margin, the position of the 28S and 18S RNA band is indicated. The GzmK transcript in CTLL-2 cells is marked by the open arrowhead.

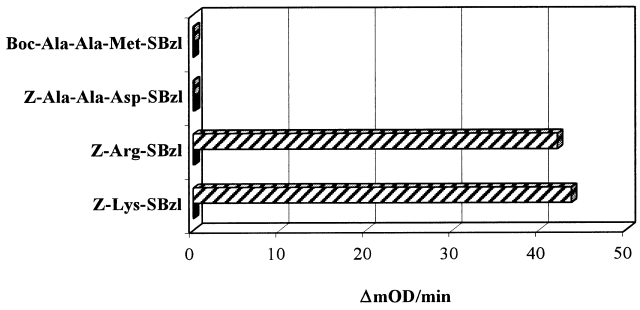

Fig. 2. Hydrolytic specificity of recombinant murine GzmK. Enzymatic activity of 5 nM mouse GzmK before (short black bars) and after conversion by cathepsin C (hatched bars) was measured using four different thiobenzylester substrates as indicated on the left in final concentrations of 0.1 mM. Increase of absorption per min (in mOD) at 405 nm was determined over a period of 5 min.

2.6. Molecular modelling of mouse bikunin

The three-dimensional structure of mouse bikunin was modelled using the known crystal structure of human bikunin as a template and the program SWISS-MODEL in the first approach mode accessible via the internet (http://www.expasy.ch/swissmod). The solvent accessible surface area of mouse bikunin was calculated with the program GRASP as described for human bikunin [10].

3. Results and discussion

3.1. Cloning of murine GzmK

Since the murine GzmK cDNA sequence was not known at the outset of this study and expressed sequence tags of the murine genome with homology to GzmK were not found in public databases, we isolated a genomic clone for murine GzmK from the genomic mouse library ICRFp703 in the vector pAD10SacBII using an almost full length cDNA probe of rat GzmK [3]. A 1.8 kb *Hin*dIII fragment of the insert was shown to contain murine GzmK exons by Southern hybridization. Subsequent sequence analysis of this *Hin*dIII fragment clearly confirmed the presence of the first two N-terminal exons by comparisons and sequence alignments with the rat GzmK cDNA sequence. The missing cDNA of exons 3 to 5 was then established by PCR and 3′ RACE from murine spleen cDNA (Clontech) as described in Section 2. Comparisons of the assembled cDNA and derived amino acid sequences with rat and human GzmK sequences [3,5,7] confirmed that this murine sequence is the closest structural homologue in the murine genome. No sequence differences were noticed between this sequence and a recently published genomic sequence for the mouse GzmK gene [2].

Expression of murine GzmK has previously been studied in various mouse tissues including bone marrow, resting spleen and thymus, but mRNA was only detected at low levels by RNA protection analyses in interleukin 2-activated lymphocytes and in cultured lymphocytes after allogeneic stimulation for 5 days [2]. We have tested several murine cell lines by Northern blotting and found moderate expression only in a CD8-positive T cell line (CTLL-2), but not in T cell lymphoma (MBL-2, LSTRA), thymoma (BW, L1210) or CD4-positive T cell lines (EL-4) (Fig. 1). Thus GzmK appears to be expressed in a more restricted manner and at lower levels than the rat and human homologues which are easily detectable in total RNA of spleen and lung.

Table 1
Inhibition of mouse and human GzmK by various inhibitors

Inhibitor	Concentration (mM)	Residual activity (%)	
		Human GzmK	Mouse GzmK
Aprotinin	0.005	6	11
Benzamidine	27.0	45	43
p-Amino-benzamidine	5.0	39	43
Leupeptin	0.2	8	78
Pepstatin A	0.001	104	93
EDTA	0.01	117	106
PefablocSC	1.0	56	6
EGR-CMK	0.1	3	48
FPR-CMK	0.1	2	0
TLCK	0.05	97	81
TPCK	0.05	118	96

Remaining activities of 4 nM mouse GzmK and 3 nM human GzmK as a percentage of initial activity were determined after 60 min of incubation at room temperature with the respective inhibitor. Every value is the average of three independent experiments.

3.2. Generation and characterization of recombinant mGzmK

Due to the low levels of murine GzmK in immune tissues and available T cell lines, it is difficult to purify sufficient amounts of mouse GzmK without contaminations by other enzymes. Previously we showed that the natural proform of human GzmK can be expressed in *E. coli*, refolded and converted into an enzymatically active enzyme by bovine cathepsin C [9]. The natural propeptide of murine GzmK, however, is Thr-Glu and was replaced by the human propeptide Met-Glu to produce the proform of murine GzmK in the cytosol of *E. coli*. By this way, murine GzmK was obtained as inclusion bodies in high yields and successfully refolded and converted into a biologically active enzyme after cathepsin C processing. Fig. 2 shows the enzymatic activity of 5 nM murine GzmK towards thiobenzylester substrates. Z-Arg-SBzl and Z-Lys-SBzl were hydrolyzed, but no other substrate. The proform of murine GzmK was catalytically inactive (Fig. 2, black columns) towards the four thiobenzylester substrates. These data clearly show that preparations of highly pure murine GzmK have Z-Lys-S-Bzl activity comparable with that of human and rat GzmK.

Recently, Z-Lys-S-Bzl activity was studied in GzmA-deficient alloreactive T cells obtained from two independent GzmA-deficient mouse strains. Hydrolytic activity for Z-Lys-S-Bzl was not detected in GzmA-deficient CTLs from

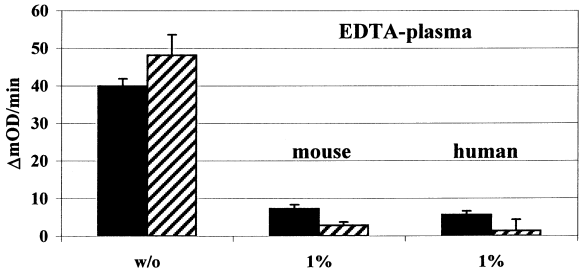

Fig. 3. Inhibition of murine GzmK by human and murine EDTA plasma in the presence and absence of 0.5 units of heparin/ml. Z-Lys-SBzl hydrolysis by 5 nM GzmK was measured after 15 min of incubation by determining the increase of absorption per min at 405 nm. GzmK activity in the absence (black bars) and presence of heparin (hatched bars) is strongly inhibited by 1% human or mouse plasma.

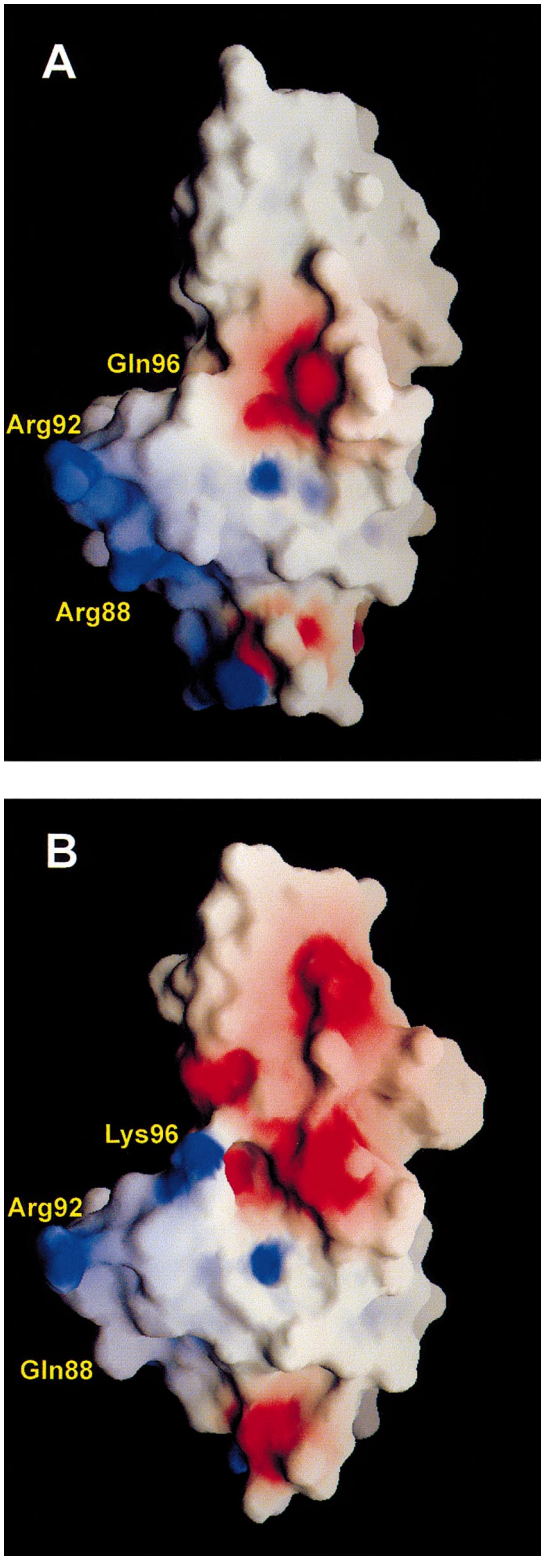

Fig. 4. Comparative representation of the accessible surface of human (A) and mouse (B) bikunin using the program GRASP. Blue indicates positive, red negative and white neutral surface potential. Primary recognition of tryptase-like target proteases occurs via the conserved Arg-92 of Kunitz domain 2. The model for mouse bikunin (panel B) was constructed with the program SWISS-MODEL in the first approach mode using human bikunin as the template.

C57Bl6 mice [8], low Z-Lys-S-Bzl hydrolyzing activity, however, in T cell populations from GzmA-deficient 129/SvJ mice [2]. These conflicting data cannot be reconciled by genetic differences at the GzmK locus, since both strains have an identical functional GzmK gene as shown in this study. Alloreactive GzmA-deficient T cells have been shown to express the message for GzmK [2], but this observation does not proof that murine GzmK has esterase activity and accounts for residual Z-Lys-SBzl hydrolyzing activity. Functional studies with pure recombinant murine GzmK were therefore performed to establish a direct link between GzmK expression and retained esterase activity in GzmA-deficient mice. Low levels of GzmK in alloreactive T cells and different T cell activation protocols are the most likely reasons why Z-Lys-SBzl esterase activity has not been observed in both GzmA-deficient mouse strains.

3.3. Functional equivalence between mouse and human GzmK

The functional equivalence between murine and human GzmK was further suggested by inhibition studies demonstrating that murine GzmK is inhibited by the same inhibitors as human GzmK including aprotinin of bovine pancreas, benzamidine and Phe-Pro-Arg-chloromethyl ketone (Table 1). The best inhibitor of human GzmK in plasma, however, is free bikunin and inter-α-trypsin inhibitor, a covalently linked protein complex between the light chain bikunin and one of the three heavy chains H1 to H3 [9]. We, therefore, tested the inhibition of mGzmK by mouse and human plasma (Fig. 3). Hundred-fold diluted mouse and human plasma inhibited mouse GzmK with high efficiency in the absence of heparin (Fig. 3). Previous experiments with inhibitors from human plasma revealed that purified recombinant bikunin in fact inhibited human GzmK at concentrations that correspond to the levels of inter-α-trypsin inhibitor in 40-fold diluted human plasma [9]. Apparent K_i values were, therefore, determined for the inhibition of mouse GzmK by human bikunin and the second Kunitz domain of bikunin and amounted to 33 and 22 nM, respectively. These values are almost identical with those apparent K_i values that we have determined for human GzmK (60 nM for bikunin and 27 nM for bikunin domain 2). Our observations imply that the surfaces of human and mouse GzmK that interact with human bikunin are highly conserved. Likewise the sites for proteinase interaction in human and murine bikunin should be highly conserved.

A three-dimensional model for murine bikunin can easily be constructed from the known crystal structure of human bikunin [10] and indicates the conservation of surface features of the two protease binding loops between positions 88 and 96 (Fig. 4A and B) and positions 111 and 116. The major differences between mouse and human bikunin domains 2 are the substitutions of Gln-96 and Gln-116 by lysine residues giving rise to a concentration of positive charges around position 96 in mouse bikunin (Fig. 4B). Conversely, Arg-88 has been replaced by a Gln residue in mouse bikunin and thus this surface region appears neutral in mouse bikunin (Fig. 4B). Since no protease in complex with bikunin has been solved to date, the conformation of the loop between positions 88 and 96 is only known for its resting state. By analogy to the complex formed between trypsin and Kunitz domains 2 of tissue factor pathway inhibitor (TFPI) [11] it is assumed that the protease recognition loop undergoes a conformational change and then binds via Arg-92 and Phe-94 tightly to its cognate enzyme [10]. Both critical residues are identical in the murine and human homologues.

Some years ago trypstatin, the second (carboxy-terminal) Kunitz domain of rat bikunin, has been reported as an efficient inhibitor for rat mast cell tryptase [12]. The human equivalent of trypstatin, however, showed no inhibition of human mast cell tryptase despite the fact that both critical residues Arg-92 and Phe-94 are also conserved in rat bikunin (H. Tschesche, personal communication). Human mast cell tryptase is known to form a tetrameric complex whose substrate binding regions are arranged around a central hydrophilic channel [13]. The geometry of this protease complex is not compatible with inhibition by bikunin or an individual Kunitz domain. In view of the striking species differences it appears unlikely that mast cell tryptases are principal targets of inter-α-trypsin inhibitor.

Binding of proteases to the second Kunitz domain of bikunin is also restrained by surface residues of domain 1 which make close rigid contacts with domain II [10]. This unique three-dimensional feature of bikunin is probably conserved in most vertebrate sequences and differs from other proteins with multiple Kunitz domains which are linked by flexible peptide spacers. Our finding that mouse GzmK is inhibited by bikunin as efficiently as human GzmK underscores the physiological relevance of this interaction and suggests that recognition sites for both inhibitors and substrates are conserved in human and rodent homologues of GzmK.

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