

Leukolysin/MMP25/MT6-MMP: a novel matrix metalloproteinase specifically expressed in the leukocyte lineage

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ABSTRACT

A novel matrix metalloproteinase (MMP) was identified from leukocytes and found to be specifically expressed by peripheral blood leukocytes among 29 different tissues examined. Named leukolysin, it encodes for 562 residues with a conserved MMP structure, i.e. pre-, pro-, catalytic-, hinge- and hemopexin-like domains, but also a RXX/RR motif, known for its role in MMP zymogen activation, and a C-terminal hydrophobic segment. Overall, leukolysin displays the strongest homology to the newly identified MT-MMP subgroup with 45% and 39% identities to MT4- and MT1-MMPs vs 30% and 31.5% to MMP1 and 3 respectively. Unlike MT4-MMP whose proteolytic activity remains undefined, a C-terminally truncated leukolysin is expressed as a strong gelatinolytic species at 28 kDa which is derived from a cell-associated 34 kDa proenzyme, presumably by furin or proprotein convertase mediated removal of the propeptide (~ 6 kDa). By green fluorescent protein (GFP) tagging, the intracellular proenzyme is localized to granules throughout the cell, suggesting that activation occur immediately prior to secretion. Taken together, leukolysin may be part of the proteolytic arsenal deployed by leukocytes during inflammatory responses.

Key words: *MT6-MMP, MMP25, leukolysin, MMP, Matrix Remodeling, Leukocytes.*

INTRODUCTION

Members of the matrix metalloproteinases (MMP) have been consistently implicated in the destruction of extracellular matrix (ECM) under many pathophysiological conditions from angiogenesis to arthritis[6,7]. To keep these powerful proteinases in latent form, most MMPs rely on a conserved cysteine in the prodomains to interact with the catalytic zinc according to the cysteine-switch latency model ADDIN ENRfu [8]. The only exception is the newly identified CA-MMP which lacks a cysteine-switch in the prodomain[9]. At the carboxyl-termini, a hemopexin-like domain is found in all MMPs except CA-MMP and matrilysin[9-11]. Recently, two additional motifs have been recognized within the MMP family: 1) a RXK/RR motif for furin or proprotein convertase (PC) mediated zymogen activation[12-14]; and 2) a hydrophobic segment at the carboxyl termini of MMP14-17 consistent with that of a transmembrane domain[15-18]. Based on these functional domains, a diverse family of MMPs must have been evolved to accomplish the tasks of ECM remodeling necessary for the growth and development of multicellular organisms such as human. In this report, a novel MMP named leukolysin was identified in leukocytes. Known for their roles in inflammatory response[4], white cells may deploy leukolysin when exiting the blood stream to reach the inflammatory sites and/or mediating the destruction of invading pathogens and infected host tissues.

MATERIALS AND METHODS

Cell lines and reagents

Mammalian cells including MDCK cells and COS 7 are cultured as described[19], [20]. Reagents associated with recombinant DNA techniques were purchased from Promega (Madison, WI). The RT-PCR/RACE kit is from Roche Diagnostics (Indianapolis, IN). Oligonucleotide primers were made by either the University of Minnesota microchemical core facility or IDT (Coralville, IA). FLAG peptide, anti-FLAG M2 monoclonal antibody and agarose beads were purchased from Sigma (St. Louis, MO).

Molecular cloning and sequence analysis of human leukolysin

Initially, EST clones (AI150017 and AA830519, both from IMAGE consortium[21]) were identified through a BLAST search to encode a putative MMP showing 60% identity to Leu235-Gly298 of MT4-MMP[18], [22]. Based on these sequences, subsequent RT-PCR and RACE reactions using RNA isolated from human leukocytes were performed to isolated a composite 2.6 kb cDNA which encodes an open reading frame. Sequencing and sequence analysis was performed

as described previously[20].

Tissue distribution of leukolysin

Human cDNA panels were purchased from Clontech (Palo Alto, CA) and amplified for the expression of leukolysin with two primers (TAC GCT CTG AGC GGC AGC and CCC ATA GAG TTG CTG CAG) at the following conditions: 2 min at 94°C for denaturation, 35 cycles of 10 sec at 94°C for denaturation, 30 sec at 52°C for annealing and 30 sec at 72°C for extension, followed by 10 min extension at 72°C. The house-keeping gene GAPDH was used as internal references as described previously[9].

Expression vectors for leukolysin1-280, leukolysin1-280-GFP and generation of their stable transfectants

The first 280 residues of leukolysin was isolated by high-fidelity PCR with pfu polymerase (15 cycles) and inserted into two vectors: pCR3.1-FLAG and pCR3.1-GFP-FLAG (Pei, etal unpublished). The 3' end of the insert was designed such that it is inserted in-frame with FLAG and GFP-FLAG respectively. The resulting vectors were sequenced and confirmed to be error-free and in-frame as designed. The resulting expression vectors were transfected into MDCK cells and stable clones were obtained by G418 selection[19]. More than 20 clones were isolated for each transfection. For screening, serum-free conditioned media from each clone were analyzed by gelatin-zymography and Western blot using M2 antibody[9]. For analysis of cell associated recombinant protein, cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 10 μ M leupeptin, 0.1 μ M 5-APMSF, 1 mM aprotinin). Cleared of any debris by microcentrifugation, the lysates were fractionated on SDS-PAGE and Western blotted with M2 antibody as described above.

Confocal microscopy

Cells were washed twice in PBS, fixed in 4% paraformaldehyde (30 min) and examined on a BioRad MRC confocal system maintained at the Biological Imaging and Processing Laboratory of the University of Minnesota.

RESULTS

Identification of a novel MMP named leukolysin/MMP25/MT6-MMP

Searching the EST database for novel MMP-like genes have resulted in the identification of two latest additions to the MMP family: CA-MMP/MMP23 and MT5-MMP/MMP24 [9],[11], [20],[23]. Continuing this effective strategy, a putative MMP gene was identified (see materials and methods), which shares considerable homology to MT4-MMP, initially reported by Puente et al[18] and recently completed by Kajita and colleagues[22]. As shown in Fig 1, this novel MMP possesses a MMP domain structure with 1) a cysteine-switch PRCXXPD in the prodomain, 2) a HEXGHXXXXXH zinc binding motif in the catalytic domain and 3) a hemopexin-like domain at the C-terminal portion. Interestingly, with a RXR/KR motif between its pro- and catalytic domains, this novel MMP apparently joins a subfamily of MMPs with a well-defined mechanism of zymogen activation mediated by members of the proprotein convertases such as furin in the trans-Golgi network

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[12],[14],[24]. At the C-terminus, a hydrophobic segment is present as a potential transmembrane domain (Fig 1b). Given its origin of isolation and tissue specific expression in leukocytes (See Fig 2), this novel gene is tentatively named leukolysin in analogy to stromelysins. Following the MMP serial designation, it is assigned MMP25 (personal communications, J.F. Woessner, Jr., University of Miami, FL). On the other hand, due to its close relationship to MT4-MMP and the other MT-MMPs, MT6-MMP is also proposed for this gene.

The relationship between leukolysin and other MMPs

To further analyze the relatedness between leukolysin and known MMPs, a multiple sequence alignment was constructed and represented in Fig 1c. Based on the alignment

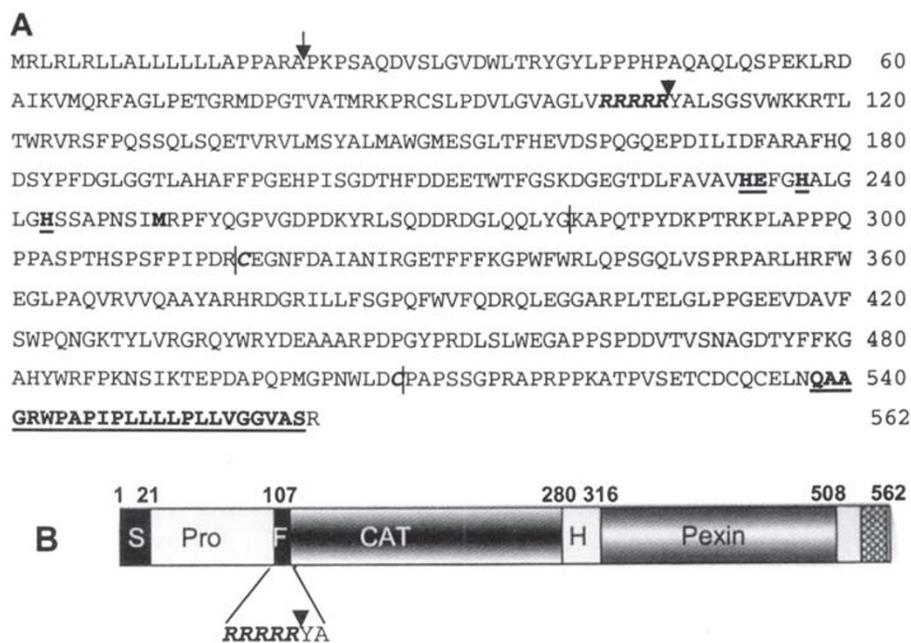


Fig 1.

Leukolysin: primary sequence, domain structure and alignments with MT-MMPs.

A. The amino acid sequence is shown with markings for the signal peptide cleavage site (downward arrow), putative zymogen activation cleavage site by furin (bold and italicized letters with a downward arrowhead), the conserved catalytic zinc binding domain (bold and underlined), M turn, the hemopexin-like domain bound by a pair of cysteines (bold and italicized), and the hydrophobic segment at the C-terminus (bold and underlined).

B. A schematic representation of domain structure for leukolysin. The numbers on the top indicate the positions of the amino acid residues bordering various domains. S: signal peptide; Pro: prodomain; F: furin recognition motif; CAT: catalytic; H: hinge; Pexin: hemopexin-like.

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MT3 -----MILLTFSTGRRL-----DFVHHSVVFLLQTLWLILCATVCGT-----E---
MT5 MPRSRGAALRRARPRAG-----ALAGPGAAALLPALCCLAAAAGAGSR-----PG---
MT2 -----MGSDPSAPGRPGWTGSLLDREEAARPLLPLLLVLLGCLGLGVA-----AED--
MT1 -----MSP-AP-RP-----SRCLLLPLLTGTALASLGSA-----QS---
MT4 --MRRRAARGPGPPPPG-----PGLSRLPLPLPLLLLLLALGTRGGCAAPEPARRA
MMP25 -----MRLRLRLAL-LLLLLAPPAR---APKPS--A

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MT3 -QYFNVEVWLQKYGYLPPDTPRMSVLRSAETMQSALAAMQQFYGINMTGKVDNRNTIDWMK
MT5 -RPFAGQNWLSYGYLLPYESRASALHSGKALQSAVSTMQQFYGIPTVTVGLDQTTIEWMK
MT2 -AEVHAENWLRLYGYLPQPSRHMSTMRSAQILASALAEMQRFYGIPTVTVGLDEETKEWMK
MT1 -SSFSPQAWLQYGYLPPGDLRTHQSRSPQSLASAAIAMQKFGYGLQVTGKADADTKAMR
MT4 EDLSLGVWLSRFGYLPADPTTQQLQTEELSKAITAMQQFGGLEATGILDEATLALMK
MMP25 QDVS LGVDWLTTRYGYLPPHPAQALQSQPEKLRDAIKVMQRFAGLPETGRMDPGTVATMR

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Furin cleavage site

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MT3 KPRCGVPDQTRGSSKFHI--RRKRYALTGQKWQHKHITYSIKNVT--PKVGDPETRKAIR
MT5 KPRCGVPDHPHLS---RRRRNKRYALTGQKWRQKHITYSIHNYT--PKVGELDRKAIR
MT2 RPRCGVPDQFGVRVKANLRRRRKRYALTGKWNHHLTFSIQNYT--EKLGWYHSMEAVR
MT1 RPRCGVPDKFGAEIKANV--RRKRYAIQGLKWQHNEITFCIQNYT--PKVGEYATYEAIR
MT4 TPRCSLPDLP-VLTQA---RRRQAPAPTCKWNRNLSWRVRTFPDRSPLGHDTVRALMY
MMP25 KPRCSLPDVLGVAGLV---RRRRYALSGSVWKKRRTLWVRVRSFPQSSQLSQETVRVLSM

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MT3 RAFDVWQVNTPLTFEEVPYSELENGK-RVDVITIIFASGFHGDSSPFDGEGGFLAHAYFP
MT5 QAFDVWQVNTPLTFEEVPYHEIKSDR-KEADIMIFFASGFHGDSSPFDGEGGFLAHAYFP
MT2 RAFRVWEQATPLVFQEVVYEDIRLRRQKADIMVLFASGFHGDSSPFDGTGGFLAHAYFP
MT1 KAFRVWESATPLRFREVPYAYIREGHEKQADIMIFFAEGFHDSTPFDGEGGFLAHAYFP
MT4 YALKVWSDIAPLNFHEV-----AGS--TADIQIDFSKADHNDGYFPDARR-HRAHAFFP
MMP25 YALMAWGMESGLTFHEVD---SPQGQ--EPDILIDFARAFHQDSYFPDGLGGTLAHAYFP

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Active site

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MT3 GPG-IGGDTHFSDPEPWTGLGNPNHDGNDLFLVAVHELGHALGLEHSSNDPTAIMAPFYQ--
MT5 GPG-IGGDTHFSDPEPWTGLGNANHDGNDLFLVAVHELGHALGLEHSSNDPSAIMAPFYQ--
MT2 GPG-LGGDTHFDADPEPWTFSSTDLHGNNLFLVAVHELGHALGLEHSSNPNAIMAPFYQ--
MT1 GPN-IGGDTHFSDAEPWTVRNEIDLNGNDIFLVAVHELGHALGLEHSSDPSAIMAPFYQ--
MT4 GHHHTAGYTHFNDDAWTFRSSDAHGMDLFAVAVHEFGHAIGLSHVAAAHSIMRPYQGP
MMP25 GEHPISGDTHFDDEETWTFGSKDGEGBDLFAVAVHEFGHALGLGHSSAPNSIMRPFYQGP

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MT3 YMETDNFKLPNDLQGIQKIYGPDPKIPPPTRPLPTVPPHRSIPPADPRKNDR-PKPPRP
MT5 YMETDNFKLPQDDLQGIQKIYGPPEAPSGATRPLPTLPPVRIHSPSE-KKHERHPRPRR
MT2 WKDVDNFKLPEDDLRGIQQLYGPDPGQPQTQPLPTVTPRRPGRPDH-----RPPRPQP
MT1 WMDTENFVLPDDDRRGIQQLYGESG-----FPTKMPPQP-RTTS-----RPSVPD--
MT4 VGDPLRYGLPYEDKVRVWQLYGVRESVSPTAQ--PEEPPLP-----EP-PDNR
MMP25 VGDPPKYRLSQDDRDLQQLYGVKAPQTPYDK--PTRKPLAP-----PPQPPAS

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MT3 P--TGRPSYP-----GAKPNICDGNFNTLAILRREMVFVKDQWFWRVRN
MT5 P--WGRPSTP-----GAKPNICDGNFNTVALFRGEMFVKDRWFWRVRN
MT2 PPPGGKPERPPKPGPPVQPRATERPDQYGNICDGFDTVAMLRGEMFVKGRWFWRVRH
MT1 -----KPKNP-----TYGNICDGNFDTVAMLRGEMFVKKRWFWRVRN
MT4 S--SAPPRK-----VPHFCSHFDAVAQIRGEAFFKGYFWRLTR
MMP25 P--THSPSFP-----IPDFCEGNFDAIANIRGETFFFKGPFWRQLQP

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MT3 NR-VMDGYPMQITYFWRGLPP---SIDAVYENS-DGNFVFFKGNKYWVFKD'TLQPGYPH
MT5 NR-VQEGYPMQIEQFWKGLPA---RIDAAYERA-DGRFVFFKGDKYWVFKVETVPEPGYPH
MT2 NR-VLDNYPMPIGHFWRGLPG---DISAAYERQ-DGRFVFFKGDYRWFREANLEPGYPQ
MT1 NQ-VMDGYPMPIQGFWRGLPA---SINTAYERK-DGKFVFFKGDKHWFDEASLEPGYPK
MT4 DRHLVSLQPAQMHRFWRGLPLHLDSVDVAVERTSDHKIVFFKGDYRWFVKDNNVEEGYPR
MMP25 SGQLVSPRPARLHRFWEGLPAQVRVVAAYARHRDGRILLFSGPQFWVQDRQLEGG-AR

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has a traditional transmembrane domain of 24 residues followed by a cytosolic domain with a conserved Valine at their termini (Fig 2a). The second group, made of MT4-MMP and leukolysin, has no visible cytosolic domains after the hydrophobic domains (Fig 1c).

Tissue specific expression of leukolysin

To investigate the distribution of leukolysin, a panel of cDNAs prepared from 26 tissues and organs were screened for leukolysin expression by PCR. As shown in Fig 2, peripheral leukocytes are the main source of leukolysin. Bone marrow and fetal kidney also express minute amounts of leukolysin, probably reflecting residual blood leukocytes in those organs at the time of sample collections.

Expression of leukolysin as a soluble enzyme

As shown in Fig 1, leukolysin possesses a hydrophobic segment which could anchor the protein on cell surface, thus, making purification of the proteinase difficult. Indeed, full-length as well as the transmembrane truncated leukolysins are expressed as cell associated forms (Pei, unpublished data). To circumvent potential problem, a vector was constructed to express leukolysin₁₋₂₈₀ tagged with FLAG epitope, without the pexin- and putative transmembrane domains (Fig 3a). Since leukolysin contains a furin site between its pro- and catalytic-domains, it is expected that leukolysin₁₋₂₈₀ will be expressed in latent form in the ER, transported to the Golgi where it is processed into active forms as described for MMP11 and MMP14[12],[13],[25]. The expression vector, pCR3.1MMP25cat, was transfected into MDCK cells and stable clones were isolated and screened by zymography. As shown in Fig 3b, a 28 kDa gelatinolytic species was detected in the conditioned media of several transfectants (lanes 3, 4 and 6). This finding is in sharp contrast to the negative results obtained when similar MT4-MMP was expressed under identical conditions (Trui and Pei, unpublished data). One of the clones with high yields, 17-13 (Fig 3b, lane 3), was chosen for further characterization.

Soluble leukolysin is secreted as a processed and fully active enzyme

With a RRRR motif, leukolysin is expected to be activated by furin or related PCs intracellularly prior to secretion. Thus, the conditioned media and cell lysates were analyzed by Western blot to establish the relationship between extracellular and intracellular leukolysins. As shown in Fig 3c, the conditioned media from 17-13 contains a major immuno-reactive species at ~ 28 kDa and a minor one at ~ 34 kDa (Fig 3c, lanes 3), potentially corresponding to the active and pro- forms of leukolysin. In contrast, more than 97% of intracellular leukolysin is in the 34 kDa form, compared to ~ 3% for the 28 kDa active forms (Fig 3c, lane 5), thus validating a precursor/product relationship for the 34 and 28 kDa species. The loss of ~ 6 kDa is presumably the result of prodomain processing at the RRRR signal by furin or its related PCs[12].

Molecular cloning of a novel MMP: MMP:25

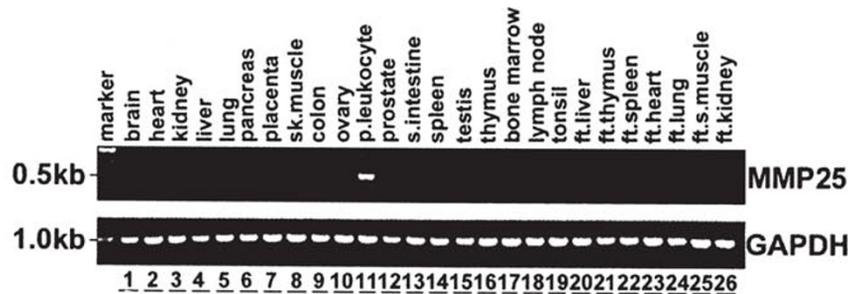


Fig 2.

Leukolysin is expressed specifically by neutrophils.

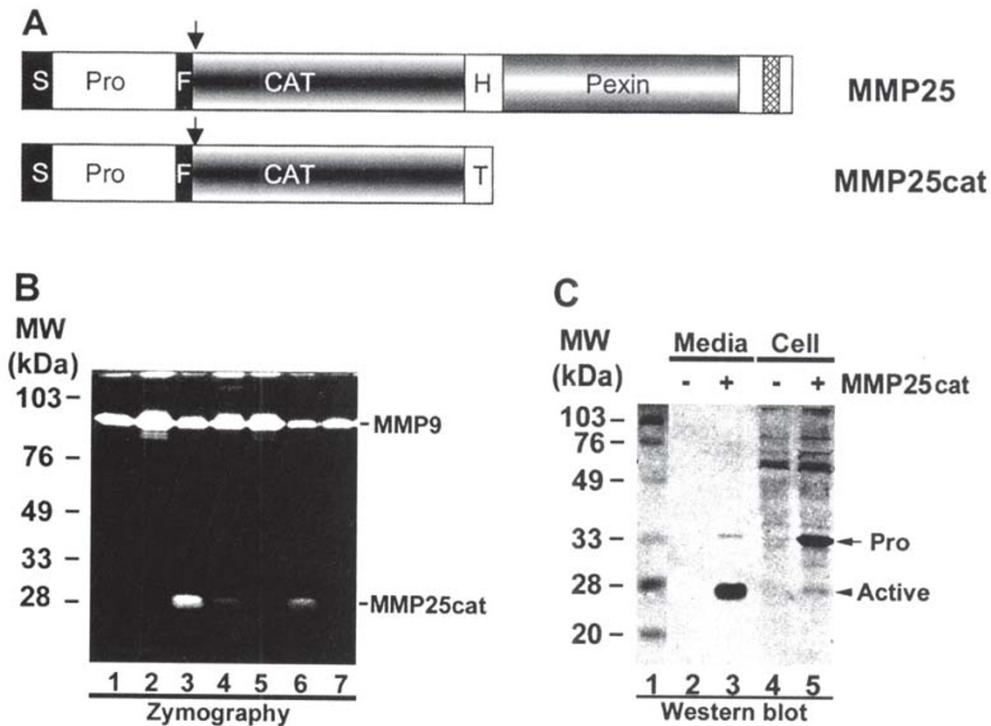
Reverse transcribed cDNA (0.5 ng aliquot each) from human multiple tissue panels (Clontech, CA) including 26 different tissues and cell types as indicated at the top (lanes 1-26) were amplified to give rise to ~ 600 bp fragment of leukolysin. To control for the amount of cDNA in each reaction, the same panels are amplified for the housekeeping gene GAPDH and presented at the lower panel.

Proleukolysin is localized in secretory granules throughout the cell

Since almost all intracellular leukolysin is in a latent state (Fig 3c), it must be residing in the trans-Golgi network waiting to be processed by furin or related proprotein convertases[12],[14]. To track the localization of proleukolysin, a GFP fusion construct was engineered to fuse leukolysin1-280 to the green fluorescent protein (GFP) (Fig 4a). Stable cell lines expressing the GFP fusion protein were generated from MDCK cells as described[19]. More than 15 positive clones were obtained and a few representatives are shown by zymography in Fig 4b. As expected, GFP adds 22 kDa to the secreted gelatinolytic species as a 50 kDa enzyme (Fig 4b). Clone 15-11 (lane 4 of Fig 4b) was selected for further analysis. Western blotting of the supernatants and cell lysates from 15-11 cells demonstrated that the majority of intracellular leukolysin::GFP is still in latent form as demonstrated for leukolysin₁₋₂₈₀ in Fig 3c (data not shown). To visualize the GFP tagged proleukolysin, confocal microscopy was performed to demonstrate that leukolysin₁₋₂₈₀::GFP is localized in secretory granules throughout the cell body, contrary to the concentrated localization in perinuclear ER and Golgi network for most secretory proteins. Thus, the signal peptide and the prodomain of leukolysin contain the necessary information sufficient to target leukolysin to secretory granules, perhaps relevant to the ones found in leukocytes[4].

DISCUSSION

As a cell type critical to inflammatory response, leukocytes are very mobile, exiting the blood stream and entering the inflamed sites to destroy invading pathogens, infected host

**Fig 3.**

Stable expression of leukolysin/MMP25₁₋₂₈₀.

A, Schematic diagram for the leukolysin expression construct. The upper portion indicates the full length leukolysin (MMP25) and the lower portion for leukolysin1-280 (MMP25cat) as an in-frame fusion with the FLAG tag as described in Materials and Methods. S: signal peptide; Pro: prodomain; F: furin; CAT: catalytic; H: hinge; Pexin: hemopexin-like; T: FLAG tag. B, A representative zymogram depicting a gelatinolytic band for leukolysin. Control (lane 1) and MMP25cat (lanes 2-7) transfected MDCK cells were stable clones and serum free supernatants from these clones were collected and analyzed by zymography as described[25]. Out of 6 clones shown, only three (lanes 3, 4, and 6) are positive for leukolysin1-280. The high molecular weight gelatinolytic species is the endogenous MMP9 from MDCK cells. Clone #17-13 was chosen for further study (lane 3). C, Analysis of intracellular and secreted leukolysin. Control (lanes 2 and 4) and MMP25cat17-13 (lanes 3 and 5) were grown to confluency and washed three times with PBS before replenished with serum free media. 48 h later, conditioned media were collected and the cells were lysed in RIPA buffer. The supernatants (lanes 1, 2) and cell lysates (lanes 3, 4) were analyzed by Western blotting using M2 antibody as described previously[9]. The pro- species is indicated by arrow and the active one by an arrowhead.

cells and damaged tissues[4]. Leukolysin fits quite naturally into the role of leukocytes as part of their proteolytic arsenals, perhaps complementing the roles of two MMPs previously found in leukocytes, neutrophils in particular: MMP8 and MMP9[26, 27]. The localization of a C-terminally truncated proleukolysin into the secretory granules of

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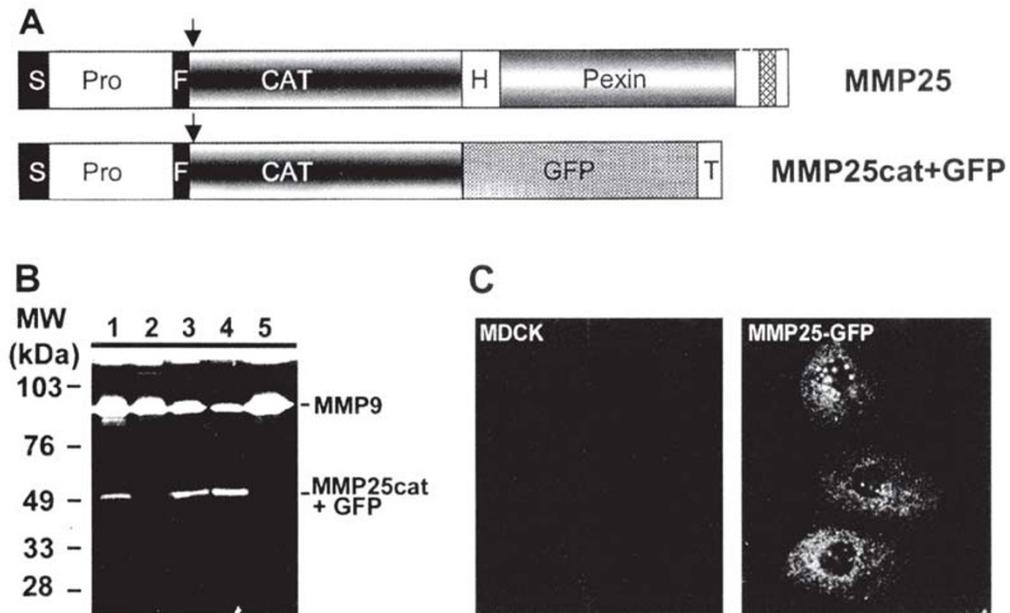


Fig 4.

Proleukolysin in secretory granules.

A, Schematic diagram of leukolysin-GFP construct. The structure of leukolysin is shown on the upper portion. Below is the in-frame fusion between leukolysin1-280 and GFP tagged with FLAG (T) (see Materials and Methods) designed to track the localization of proleukolysin inside the cells. **B**, a representative zymogram of stable clones transfected with MMP25cat-GFP. Control (lane 5) and leukolysin-GFP transfectants (lane 1-4) were grown to confluency and conditioned with serum free media which were subsequently analyzed as described in Fig 3. GFP contributes about 22 kDa to the molecular weight of the fusion protein (50 vs 28 kDa, see Fig 3). **C**, Confocal localization of Leukolysin/MMP25cat-GFP. The left panel is confocal image of control transfected cells from lane 5 of panel B. The right panel is for cells transfected with MMP25cat-GFP (shown here is clone 15-11, lane 4 of panel B, but all clones behave identically) sectioned optically on a BioRad MRC confocal system. The intracellular proleukolysin is localized mainly in secretory granules throughout the cell bodies.

MDCK cells seems to suggest that leukolysin may be stored as zymogens in the intracellular granules of leukocytes-a depot of many powerful, yet latent, proteinases[4]. Although characterized as a potent proteinase against denatured collagen (Fig 3b), leukolysin's role in ECM destruction is not known at present. With the availability of purified enzymes and the full-length cDNA clone, the full potential of leukolysin as an ECM degrading enzyme is under investigation thoroughly. Furthermore, a genomic clone

has been isolated from mouse SVJ129 genomic library (Yi and Pei, unpublished data). Given its tissue specific nature of expression, leukolysin deficient mice generated by genetic ablation should display functional deficiency in leukocyte- or inflammation related physiological as well as pathological processes such as inflammatory responses.

With a RRRR motif, leukolysin is apparently a new member of a MMP subgroup that can be activated by furin-type proprotein convertases[12],[24]. Although the precise mechanism for its activation remains to be investigated, proleukolysin (34 kDa) was shown to be processed intracellular into the 28 kDa active species (Fig 3c), presumably by furin or related PCs. MMP activation mediated by furin or related proprotein convertases was first proposed during the characterization of stromelysin-3[12]. With the recent identification of leukolysin/MMP25, MT5-MMP/MMP24 and CA-MMP/MMP23, close to 40% of known MMPs (8 out of 20) possess a RXK/RR motif, ready to be cleaved and activated by furin or related PCs[5],[9],[11], [20],[23],[24]. Addition of leukolysin to this group further illustrates the importance of furin- or PC- mediated MMP activation in ECM remodeling processes.

By displaying the strongest homology to the MT-MMP subgroup, especially MT4-MMP (Fig 1c), leukolysin is also named MT6-MMP. Based on the alignment in Fig 1c, it seems plausible to divide the MT-MMPs into two branches: MT1, 2, 3, 5-MMPs and MT4, 6-MMPs. The defining function for the former group is their ability to activate progelatinase A[15],[16],[20], whereas the later can not. Current efforts are underway to address the roles of signal peptide and prodomain in targeting proleukolysin to intracellular granules (Fig 4) and that of the C-terminal hydrophobic domain in cell membrane localization. Anchored on cell surface, active leukolysin could be in close contact with substrates and secluded from the anti-proteinase shield in the extracellular milieu, thus, may exert a unique force in destroying and remodeling extracellular matrix during leukocyte-mediated inflammatory responses.

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