FEBS Letters 580 (2006) 2227–2232

Inhibition of human matriptase by eglin c variants

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Received 2 February 2006; revised 2 March 2006; accepted 9 March 2006

Available online 20 March 2006

Edited by Michael R. Bubb

Abstract **Based on the enzyme specificity of matriptase, a type II transmembrane serine protease (TTSP) overexpressed in epithelial tumors, we screened a cDNA library expressing variants of the protease inhibitor eglin c in order to identify potent matriptase inhibitors. The most potent of these, $R_1K'_4$-eglin, which had the wild-type Pro^{45} (P1 position) and Tyr^{49} (P4′ position) residues replaced with Arg and Lys, respectively, led to the production of a selective, high affinity (K_i of 4 nM) and proteolytically stable inhibitor of matriptase. Screening for eglin c variants could yield specific, potent and stable inhibitors to matriptase and to other members of the TTSP family.**

Keywords: Matriptase; Eglin c; Serine protease; Enzyme inhibition

1. Introduction

The type II transmembrane serine proteases (TTSPs) are an emerging class of cell surface proteolytic enzymes. Matriptase, one the most extensively studied member of this family, has the broadest expression pattern of all the TTSPs, being detected in a wide range of human tissues [1]. Although studies of knockout mice have revealed that matriptase is involved in the development of the epidermis, hair follicles, and cellular immune system [2], very few in vivo substrates for this enzyme have been identified. It has been suggested that matriptase could play important roles in tumor cell metastasis and invasiveness [3–9]. Indeed, this enzyme causes malignant transformation when orthotopically overexpressed in the skin of mice thus suggesting the causal role of matriptase in human carcinogenesis [9]. Other members of the TTSPs, such as Tmprss2 [10], Tmprss3 [11] and hepsin [12], have also been implicated in cancer. Thus, matriptase, and the TTSPs in general, are potentially interesting therapeutic targets.

Recently, it was demonstrated that potent inhibitors for furin and kexin, members of the proprotein convertases family [13], could be produced by introduction of furin or kexin recognition sequences within the reactive site of the protease inhibitor eglin c [14,15]. Further studies based on the three-dimensional structure of eglin c have found that optimization of interaction between enzyme and inhibitor could be addressed by screening eglin c cDNA libraries containing randomly substituted residues within projected adventitious contact sites outside the reactive site [16]. The similarity in specificity between matriptase and furin, which preferentially recognizes the R–X–X–R (P4 to P1 position) sequence [17], led us to the hypothesis that matriptase and other members of the TTSP family could be targets of genetically engineered variants of serine protease inhibitors such as eglin c.

In the present study, we were able to produce eglin c variants that exhibit strong and selective inhibition against matriptase by mutagenesis of amino acids in and outside the inhibitor's reactive site loop. We demonstrate that the eglin c protein could be used as a macromolecular scaffold to develop potent and selective inhibitors for matriptase and to a larger extent for all the members of the expanding TTSP family.

2. Materials and methods

2.1. Materials

Matriptase cDNA was a generous gift from Dr. Chen-Yong Lin (Georgetown University, Washington, USA). Purified recombinant human airway trypsin-like protease (HAT) was a generous gift from Dr. Manabu Chokki (Teijin Pharma Limited, Tokyo, Japan). Wild-type synthetic eglin c (termed L_1-eglin), R_1-eglin, K_2R_1-eglin and R_4R_1-eglin cDNA constructions as well as the Y49X-eglin cDNA library were a generous gift of Dr. Robert S. Fuller (University of Michigan, Ann Arbor, USA).

2.2. Construction, expression and purification of matriptase 596–855

Nucleotides corresponding to amino acids 596–855 of matriptase were amplified by PCR, ligated into the pQE-30 vector and transformed in *Escherichia coli* M15 strain (Qiagen, Mississauga, Ont., Canada). Expression was induced for 4 h at 37 °C with 0.5 mM IPTG (Promega, Madison, WI, USA). Bacterial cells were harvested, resuspended in lysis buffer (50 mM Tris–HCl, pH 8, 500 mM KCl, 10% glycerol, 1 mM β-mercaptoethanol), sonicated and centrifuged at $10\,000 \times g$ for 15 min. The pellet was solubilized in 6 M urea. Proteins were purified using immobilized metal-chelate affinity chromatography (IMAC). Fractions were pooled and dialysed overnight at 4 °C against 50 mM Tris (pH 9), 10% glycerol, 3 M urea and 1 mM β-mercaptoethanol. Proteins were dialysed for 6 h at 4 °C against 50 mM Tris (pH 9), 10% glycerol, 1 mM β-mercaptoethanol. Matriptase was further purified using Mono-Q ion exchange chromatography on an Explorer system (Amersham Biosciences, Baie d'Urfé, Que., Canada).

2.3. General kinetic methods

Enzyme activities were monitored by measuring the release of fluorescence from AMC-coupled peptides (excitation, 360 nm; emission, 441 nm) in a FLX-800 TBE microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The purified human matriptase was active-site titrated with the burst titrant 4-methylumbelliferyl-*p*-guanidino

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Abbreviations: TTSP, Type II transmembrane serine protease; IMAC, immobilized metal-chelate affinity chromatography; MUGB, 4-methylumbelliferyl *p*-guanidino benzoate; HAT, human airway trypsin-like protease; AMC, 7-amino-4-methylcoumarin

0014-5793/$32.00
doi:10.1016/j.febslet.2006.03.030

benzoate (MUGB). Enzymatic assays with matriptase were performed in Tris–HCl 100 mM containing 500 μg/ml BSA at pH 9. Human soluble furin was expressed, purified, titrated and assayed as described previously [18]. The purified HAT protein was active-site titrated with MUGB. Assays with HAT were performed in Tris–HCl 50 mM at pH 8.6.

2.4. Construction, expression and purification of eglin c variants

Wild-type synthetic eglin c protein, (also termed L_1-eglin in Ref. [14] or WT-eglin herein), R_1-eglin, K_2R_1-eglin and R_4R_1-eglin constructions were described previously [14]. In essence, WT-eglin is a protein product produced from a synthetic gene optimized for prokaryotic expression where the three N-terminal residues (Met^1-Ser-Met) differ in sequence from that of the naturally occurring eglin c (Thr^1-Gly-Phe). All eglin c variants studied herein are protein products of the optimized (Met-Ser-Met) construct. $R_4R_1E'_4$-eglin, $R_4R_1K'_4$-eglin and $R_4R_1R'_4$-eglin were recovered from the rapid screening of matriptase inhibition by the Y49X library (see below). $R_1K'_4$-eglin was obtained by substitution of the tyrosine 49 for a lysine in the R_1-eglin construction using the Quickchange II mutagenesis kit (Stratagene, La Jolla, CA, USA) as described by the manufacturer. All eglin c variants were transformed into bacterial host strain BL21-(DE3) pLys (Novagen, Madison, WI, USA) and the expression and purification was performed as described [14].

2.5. Screening of eglin c Y49X library

A cDNA eglin c variant library, in which all three nucleotides of the codon corresponding to residue 49 were fully randomized [16], was used to screen for matriptase inhibition. This library, termed Y49X, was transformed into bacterial host strain BL21-(DE3) pLys. Transformants were grown in 200 μl of LB containing kanamycin (50 μg/ml) at 37 °C in 96-well, 0.45-μm GHP membrane filter plates (Pall Life Sciences, Ville St.-Laurent, Que., Canada). When OD_{600} values reached 0.4, expression was induced for 4 h with 1 mM IPTG in the filter plate (before IPTG addition, 60 μl of culture was removed and transferred to a 96-well master plate in which 60 μl of a 50% glycerol solution was added to each well for storage at −80 °C). Purification of eglin c variants was performed as described [16]. Partially purified eglin c variants (10 μl) were pre-incubated with matriptase (4 nM) for 15 min at room temperature in a 96-well plate (Corning, Acton, MA, USA). Residual matriptase activity was recorded after 30 min of incubation with Boc-Glu-Glu-Arg-AMC. Vectors encoding selected variants were recovered from master plates and sequenced. Interesting variants were purified to homogeneity (as described earlier) on a 50 ml scale for further characterization.

2.6. Inhibition assays

Enzymes were diluted to concentration ranging from 4 to 12.5 nM for furin, from 2 to 7 nM for matriptase and 20 pM for HAT and incubated with appropriate dilutions of the eglin c variants for 15 min at room temperature. Residual enzyme activity was measured by following the hydrolysis of a fluorogenic substrate (4 μM Boc-Arg-Val-Arg-Arg-AMC for furin and 4 μM Boc-Val-Pro-Arg-AMC for matriptase and HAT) (Bachem Bioscience, King of Prussia, PA, USA). Data from three independent experiments or more were averaged and residual velocities were plotted as a function of eglin c concentration. Data were fitted by non-linear regression analysis to the Eq. (1) [19] using the Enzfitter software (Biosoft, Ferguson, MO, USA)

$$v_i/v_o = 1 - \{([E]_0 + [I]_0 + K_{i(app)}) - (([E]_0 + [I]_0 + K_{i(app)})^2 - 4[E]_0[I]_0)^{1/2}\}/2[E]_0, \quad (1)$$

where v_0 and v_i are the steady-state rates of substrate hydrolysis in the absence and presence of inhibitor, respectively, $[E]_0$, the initial concentration of enzyme, $[I]_0$, the initial concentration of inhibitor and $K_{i(app)}$ the substrate-dependent equilibrium dissociation constant. The substrate-independent constant K_i was calculated using the Eq. (2) [19],

$$K_i = K_{i(app)}(1 + [S]_0/K_m), \quad (2)$$

where $[S]_0$ is the initial concentration of substrate and K_m the Michaelis–Menten constant for the enzyme–substrate interaction. To investigate the stability of the eglin c variants, 4 μM of inhibitor was incubated at room temperature with the indicated concentration of matriptase or HAT for the indicated time. Proteins were then resolved by SDS–PAGE and revealed using the Gel Code blue stain reagent (Pierce Biotechnology, Rockford, IL, USA).

2.7. Molecular modeling

The complex between eglin c and matriptase was based on the experimental structures of the chymotrypsin/eglin c complex (PDB ID-1ACB) and of the matriptase/BPTI complex (PDB ID-1EAW). The primary structures of matriptase (from 1EAW) and of chymotrypsin (from 1ACB) were aligned with 43% identity. The structurally conserved region (SCR) encompassing residues Ile16 and Cys58 of chymotrypsin and Val16 to Cys58 of matriptase (near the S1 subsite) showed 56% identity. 3D superimposition of this SCR gave a root mean square deviation (RMSD) of 1.3064 Å between the Cα of both enzymes. The overall structure is very similar for both enzymes and the residues forming the catalytic triad (Ser195, His57 and Asp102) are positioned in the matriptase active site exactly as in chymotrypsin. Superimposition of residues P3 to P4′ of eglin c (i.e., Val43 to Tyr49, from 1ACB) and BPTI (Pro13 to Ile19, from 1EAW) gave a RMSD value of 0.5073 Å. Hence, the structures of matriptase (from 1EAW) and eglin c (from 1ACB) were assembled into one complex, and hydrogen atoms were added. This WT complex was subjected to 1000 steps of energy minimization (all atoms were free to move) using conjugate gradients (CG) with a dielectric value of 78. After the minimization process, the RMSD between the heavy atoms of matriptase and the initial 1EAW structure was 1.2749 Å and between eglin c and the initial 1ACB structure was 0.8859 Å. In order to study the effect of mutations at P1 and P4′ positions, concerned residues were replaced in the model (i.e., Leu45Arg, Tyr49Lys or Tyr49Glu) and each resulting complex was subjected to another 1000 steps of minimization using CG. Molecular rendering was realized with ribbons [20].

3. Results

3.1. Expression, purification and characterization of human matriptase

The matriptase construct (amino acids 596–855) used in this study encompassed the enzyme's activation and catalytic domains. The protease was expressed in *E. coli* as a His-tagged fusion protein and was purified from denatured inclusion bodies using IMAC. Denatured matriptase was then refolded by a 2-step dialysis procedure to gradually remove the denaturant. The refolded zymogen, which migrates at 29 kDa, was capable of auto-activation as demonstrated by the decrease in the size of the protein to 26 kDa (Fig. 1A and B). Fully active matriptase was previously shown to occur from autoproteolytic cleavage of its activation domain [1].

Interestingly, the redox environment was important for proper folding of matriptase. Addition of 1 mM β-mercaptoethanol in the refolding buffer was essential to obtain a proteolytically active protease (data not shown). The presence of the activation domain was also a pre-requisite for the production of an active protease. Thus, the expression and purification of a protein containing exclusively the catalytic domain of matriptase (amino acids 615–855) produced an inactive enzyme (data not shown). To examine the pH profile of the purified enzyme, we assayed for matriptase activity between pH 6 and pH 11 using Boc-Glu-Ala-Arg-AMC as a substrate. The results indicate that purified matriptase has optimal activity from pH 9 to pH 11 (data not shown). In the ensuing experiments, matriptase activity was measured at pH 9.

3.2. Inhibition of recombinant matriptase by an eglin c variant

Furin has previously been shown to be inhibited by an eglin c variant protein, R_4R_1-eglin, with substituted P_{42} and L_{45} for

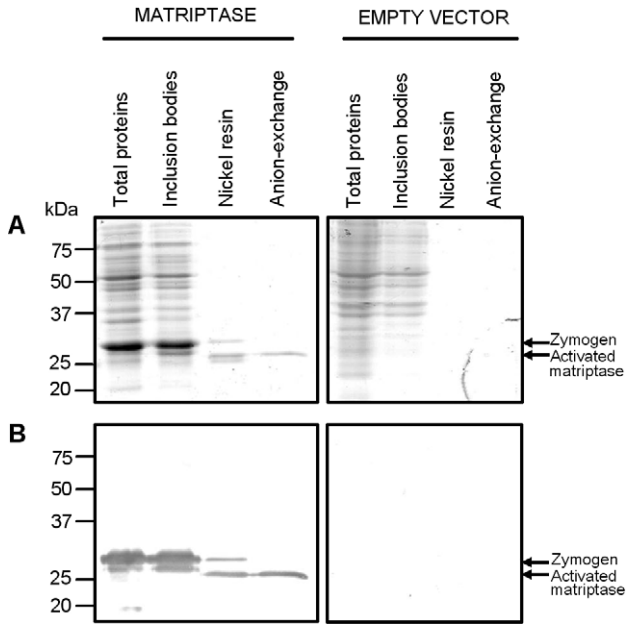

Fig. 1. Expression of matriptase from *E. coli* during different stages of purification. Expression of matriptase was induced in *E. coli* transformed either with matriptase construct or empty pQE-30 vector with 0.5 mM IPTG for 3 h at 37 °C. Samples for each step of purification (total proteins, inclusion bodies, renatured nickel-charged resin purified proteins, mono-Q FPLC purified proteins) were resolved by SDS–PAGE. (A) Coomassie blue staining of total proteins. (B) Western blot analysis was performed with rabbit anti-human matriptase and donkey anti-rabbit HRP antibodies (Bethyl laboratories, Montgomery, TX, USA).

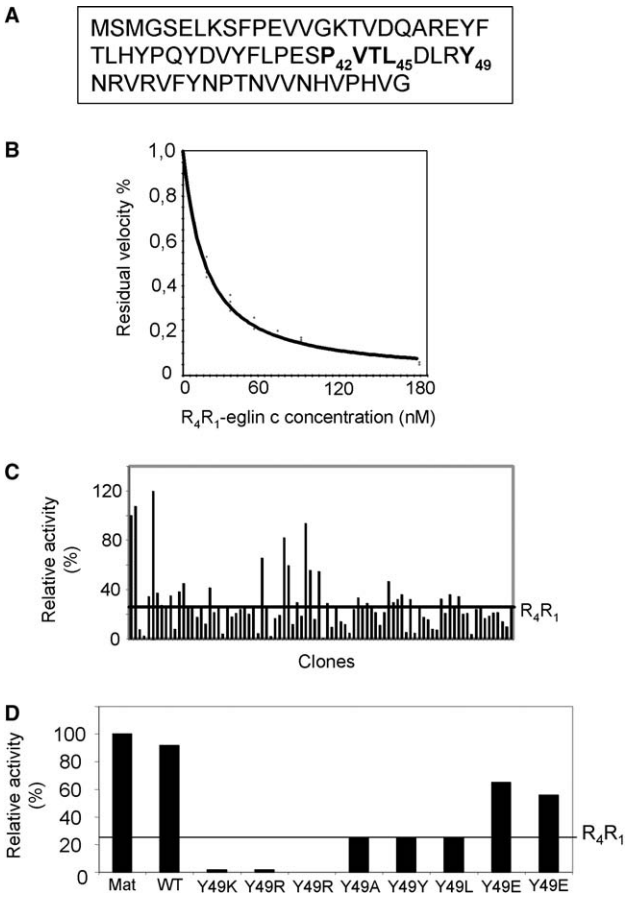

Fig. 2. Inhibition of matriptase by eglin c variants. (A) Amino acid sequence of the "wild-type" eglin c protein (L_1-eglin) used in this study [14]. The protein is a product of a synthetic eglin c gene used to optimize expression in *E. coli*. Reactive-site loop (P4 to P1) and tyrosine-49 residue, corresponding to position P4′, are shown in bold. (B) Inhibition assay between matriptase and R_4R_1-eglin as described in Section 2. (C) Matriptase inhibition by eglin c variants in a 96-well plate. Each bar represents an eglin c variant with a residue in position 49 that has been randomly substituted for any one of the 20 possible amino acids. The horizontal bar represents the level of inhibition by R_4R_1-eglin. (D) Selected clones were identified by automated sequencing. Substitutions are identified below the clone number. Mat: Matriptase activity without inhibitors. WT, level of matriptase inhibition by wild-type eglin c. The horizontal bar represents the level of inhibition by R_4R_1-eglin.

arginine residues (R4 and R1 correspond to P4 and P1 positions, respectively, according to the nomenclature of Schechter and Berger [21]) but not by wild-type eglin c (for amino acid sequence see Fig. 2A) [14]. Incubation with R_4R_1-eglin abolished matriptase activity while the wild-type eglin c had no effect (data not shown). To characterize the inhibition properties of R_4R_1-eglin towards matriptase, inhibition assays were performed and a K_i of 26 nM was determined (Fig. 2B and Table 1). R_4R_1-eglin was also a good inhibitor for furin with a K_i of 62 nM, but a weak inhibitor of human airway trypsin-like protease (HAT), another member of the TTSP family [22], with a $K_i > 1000$ nM.

3.3. Optimization of matriptase inhibition by substitution of position 49 of eglin c

Position 49 of eglin c, occupied by Tyr and corresponding to the P4′ position, had already been shown to be implicated in the selectivity and affinity of the inhibitor towards furin, PC7 and Kex2 [16]. In order to verify the effect of the P4′ position on matriptase activity within a R_4R_1-eglin background, a cDNA library of R_4R_1-eglin variants where each one of the 20 amino acids are substituted at position 49 was screened for matriptase inhibition (Fig. 2C). Selected variants that appeared to give enhanced, decreased or unchanged inhibition relative to the control (R_4R_1-eglin) in an initial rapid screen were sequenced at the nucleotide level (Fig. 2D). R_4R_1-eglin variants that were found to give enhanced inhibition contained an Arg or Lys at position 49 instead of the original Tyr residue. Variants that showed no changes in matriptase inhibition contained either Ala, Leu or Tyr at position 49. Finally, the two selected R_4R_1-eglin variants that showed decreased inhibition properties contained a Glu at position 49.

K_i values for the interaction between furin, matriptase or HAT and three variants of R_4R_1-eglin substituted at position 49 were determined and compared (Table 1). $R_4R_1K'_4$-eglin exhibited a 4-fold higher affinity for matriptase than R_4R_1-eglin with a K_i of 6.1 nM. In contrast, substitution for Glu in position 49 ($R_4R_1E'_4$-eglin) resulted in an inhibitor exhibiting an affinity 5-times lower for matriptase than R_4R_1-eglin with a K_i of 140 nM while $R_4R_1R'_4$-eglin had a K_i of 11 nM. These three eglin c variants appeared to be only weak inhibitors of HAT ($K_i > 1000$ nM). In contrast to matriptase, $R_4R_1R'_4$-eglin but also $R_4R_1E'_4$-eglin showed improved affinity for furin with K_i of 29 nM and 27 nM, respectively, while $R_4R_1K'_4$-eglin showed similar affinity for furin with a K_i of

Table 1
K_i values for the different eglin c variant proteins

Inhibitor	Sequences P_4–P'_4	K_i (nM)		
		Furin	Matriptase	HAT
	↓			
Eglin WT	PVTLDLRY	nd	>1000	nd
R_1-eglin	PVT**R**DLRY	>1000	18	420
$R_1K'_4$-eglin	PVT**R**DLR**K**	>1000	4.5	>1000
K_2R_1-eglin	PV**KR**DLRY	630	930	>1000
R_4R_1-eglin	**R**VT**R**DLRY	62	26	>1000
$R_4R_1R'_4$-eglin	**R**VT**R**DLR**R**	29	11	>1000
$R_4R_1K'_4$-eglin	**R**VT**R**DLR**K**	94	6.1	>1000
$R_4R_1E'_4$-eglin	**R**VT**R**DLR**E**	27	140	>1000

Eglin c variant proteins were obtained by substitution of residues corresponding to amino acids 42–49 (P4–P4′ position). Sequence for each variant from amino acids 42–49 is indicated and substitution are shown in bold. K_i values were determined as described in Section 2. Values represent an average of at least three independent experiments. Experimental errors were ±25 or less. ND, not determined (very weak inhibitor).

94 nM. The specificity of $R_4R_1K'_4$-eglin was greatly enhanced when compared to R_4R_1-eglin with a 15-fold higher selectivity for matriptase than furin. Specificity of $R_4R_1E'_4$-eglin was also enhanced with a 5-fold higher selectivity for furin than matriptase. These results indicate that the most selective and potent eglin c variant is $R_4R_1K'_4$ for matriptase.

3.4. Substitution within the reactive site loop of eglin c creates variants with enhanced specificity and stability

We next decided to investigate whether amino acids inside the reactive site loop of eglin c could be substituted to obtain even more potent inhibitors of matriptase than $R_4R_1K'_4$-eglin. We tested the ability of R_1-eglin (position P1) and K_2R_1-eglin (position P2 and P1) to inhibit matriptase hydrolytic activity. R_1-eglin showed similar affinity (18 nM) for matriptase than R_4R_1-eglin while K_2R_1-eglin showed a dramatic decrease in affinity compared to R_1-eglin or R_4R_1-eglin (Table 1). R_1-eglin had a 23-fold higher affinity for matriptase than HAT and was a weak inhibitor of furin. Because the introduction of a lysine instead of tyrosine in position 49 of the R_4R_1-eglin variant resulted in an improved inhibitor for matriptase, we decided to substitute the Tyr49 of R_1-eglin for a lysine. The produced $R_1K'_4$-eglin inhibitor, when compared to R_1-eglin, showed a 4-fold enhanced affinity for matriptase with a K_i of 4.5 nM. $R_1K'_4$-eglin is only a weak inhibitor of furin and HAT (K_i of >1000 nM) (Table 1).

Stability of the different eglin c variants was investigated in the presence of active matriptase. R_4R_1-eglin, K_2R_1-eglin, $R_4R_1K'_4$-eglin, $R_4R_1R'_4$-eglin, $R_4R_1E'_4$-eglin were all cleaved when incubated with matriptase for 5-hour using an enzyme:inhibitor ratio of 1:4 (Fig. 3A and B left panel). Conversely, $R_1K'_4$-eglin and R_1-eglin showed virtually no cleavage after a 5 h incubation under these conditions (Fig. 3B, right panel). These results indicate that R_1-eglin variants are better inhibitors of matriptase than R_4R_1-eglin variants with equally high affinity but enhanced selectivity and stability. Overall, $R_1K'_4$-eglin proved to be the best matriptase inhibitor with regards to its affinity, selectivity and stability.

4. Discussion

Native and genetically engineered eglin c variants had previously been showed to be efficient inhibitors of several serine proteases including the human leukocyte elastase [23], chymotrypsin [24], trypsin [25] and members of the pro-protein convertases family [14–16,26]. This inhibitor, as a member of the potato inhibitor 1 family, has a flexible reactive site loop that increases its ability to adapt for enhanced interaction and inhibition of new target enzymes [27,28]. Here we show that simple substitutions in the reactive site loop of eglin c, yield high affinity inhibitors (low nanomolar) to matriptase. The simple introduction of an arginine residue in the P1 position of the original P4-P1 target sequence of eglin c, Pro-Val-Thr-Leu, trans-

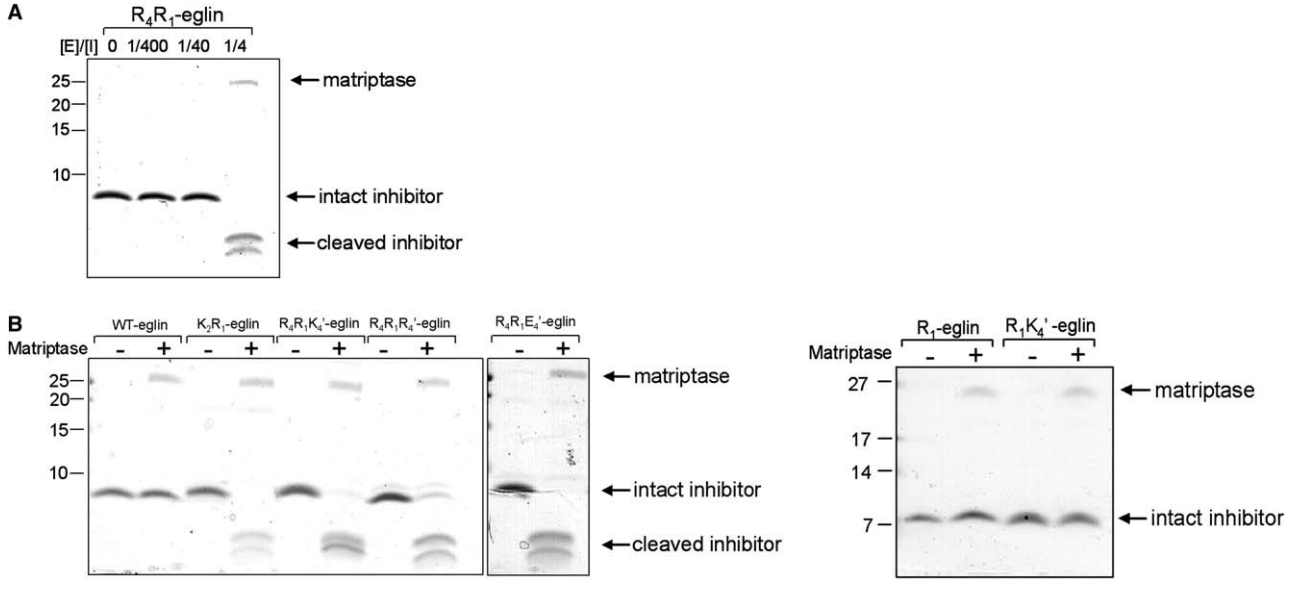

Fig. 3. Susceptibility of the eglin c variants to matriptase cleavage. (A) 4 μM of R_4R_1-eglin was incubated with the indicated quantity of matriptase for 5 h at 21 °C before electrophoresis and revelation. [E]/[I] corresponds to the stoichiometric enzyme/inhibitor ratio. (B) 4 μM of eglin c variants were incubated without (−) or with (+) 1 μM of matriptase for 5 h at 21 °C before electrophoresis and revelation.

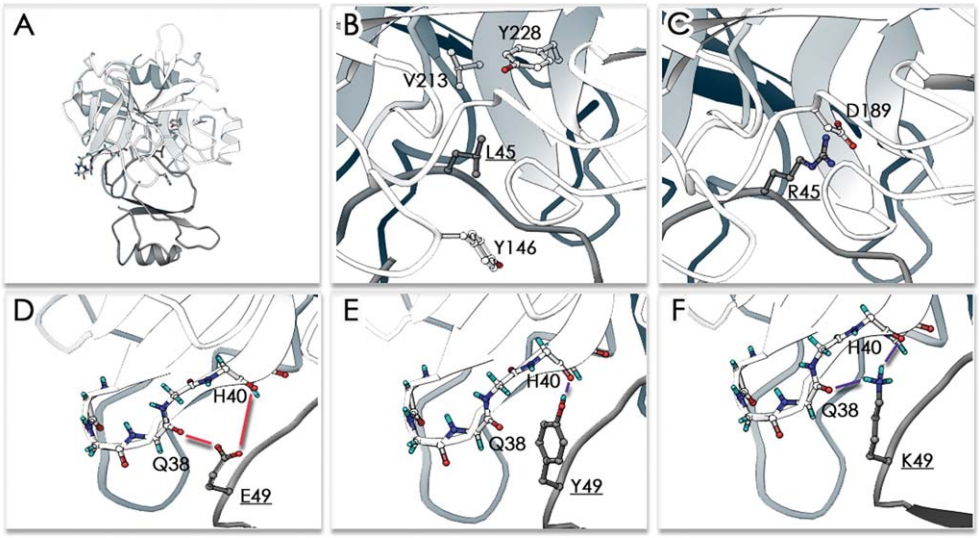

Fig. 4. Molecular modeling of the matriptase/eglin c complex. (A) The matriptase structure is shown in white (faded to blue), whereas eglin c is in dark gray. Oxygen atoms are shown in red, nitrogen in blue and hydrogen in cyan. Matriptase residues were numbered according to Friedrich et al. [29]. Eglin c residues are underlined. (B–F) Close up on positions P1 and P4′ of eglin c and their interacting counterparts in matriptase. (B) In the WT eglin c, residue Leu45 forms favorable hydrophobic interactions with residues Tyr146, Val213 and Tyr228 at the S1 subsite of matriptase. (C) When an Arg residue is present at P1, its charged guanidinium group forms a salt bridge with the carboxylate of Asp189 in S1. (D) The presence of the negatively charged Glu residue at P4′ impairs the formation of stabilizing hydrogen bonds with the backbone of Gln38-His40 in matriptase. Red bars indicate unfavorable interactions. (E) In WT eglin c, the hydroxyl group of Tyr49 at P4′ allows the formation of a stabilizing hydrogen bond with the carbonyl of Gln38, which can be interchanged with the carbonyl of His40. Blue bars indicate favorable interactions. (F) Replacement of Tyr49 by a Lys residue allows the formation of 2 stabilizing hydrogen bonds between the positively charged ε-ammonium group and the backbone carbonyls of Gln38 and His40. Blue bars indicate favorable interactions.

formed a weak inhibitor in a potent one. Furthermore, substitution at the P4′ position yielded an even more potent inhibitor of matriptase. The importance of the P4′ position in the recognition of eglin c by the members of the pro-protein convertases family had been previously noted [16]. For matriptase, it is very interesting to note that introduction of a basic residue (arginine or lysine) at this position had a positive effect on the recognition of eglin c variants by matriptase while the introduction of an acidic residue (glutamic acid) had a negative effect. The best inhibitor of matriptase in our study, $R_1K'_4$-eglin, is also resistant to proteolysis which is not the case for the R_4R_1-eglin variants. Thus, the $R_1K'_4$-eglin showed remarkable inhibition characteristics with a high affinity for matriptase and high stability. We used molecular modeling to gain insights into the eglin c's structure-activity of positions P1 and P4′ with regards to matriptase (Fig. 4A-F). The S1 subsite of matriptase contains a few hydrophobic residues, namely Tyr146, Val213 and Tyr228, which can form stabilizing interactions with hydrophobic residue at P1 such as Leu45 in eglin c (Fig. 4B). On the other hand, and as previously reported [29], the presence of Asp189 within S1 allows the formation of a favorable salt bridge with a positively charged residue at P1 (Fig. 4C). The favorable electrostatic interaction formed with an Arg at P1 is more important in the stabilization energy of the complex than the hydrophobic contacts observed with a Leu residue at P1, leading in turn to a lower K_i for R_1-eglin mutant than for WT eglin c. Interactions between position P4′ of eglin c and matriptase are less specific as they involve only backbone atoms of the enzyme. The complex is less stable when Tyr49 is replaced by a negatively charged Glu residue, because no favorable hydrogen bond can be formed with the enzyme (Fig. 4D). The hydroxyl group of Tyr49 at P4′ can form one hydrogen bond with the backbone carbonyl of either Gln38 or His40 (Fig. 4E). Replacement of Tyr49 by a positively charged Lys residue allows the formation of two stabilizing hydrogen bonds with backbone carbonyls of Gln38 and His40 and thus enhances stabilization of the complex compared with WT eglin c (Fig. 4F).

Because of the suggested roles of matriptase in tumor invasiveness and metastasis, several groups have been interested in the development of potent matriptase inhibitors through the use of different strategies, such as development of small molecule inhibitors [30,31], mutation of the binding interface of ecotin [32] and phage-displayed antibody library screening [33]. Optimization of eglin c variants-matriptase contacts by in vitro molecular evolution provides an additional strategy to generate specific inhibitors of matriptase. TTSPs are an expanding family of extracellular proteases and their specific function is still poorly understood. Despite the fact that the eglin c variants used in this study were not suitable inhibitors of recombinant HAT, the use of an eglin c macromolecular scaffold to produce specific inhibitors for other members of the TTSPs could be envisioned. Eglin c inhibitors display interesting characteristics in that they are small, make stable enzyme–inhibitor complexes and lack disulfide bridges for easy expression in bacterial cultures. All these properties could make them particularly useful tools in a diverse range of future investigations, from the elucidation of matriptase's physiological function at the cell membrane to the potential production of potent and specific therapeutics.

Acknowledgments: J.-M.L. was supported by a Fonds de la Recherche en Santé du Québec (FRSQ) studentship award. R.L. is a "Chercheur-National" scholar of the FRSQ. We are especially grateful to

P. Lavigne for discussions and comments on the modelling of the matriptase/eglin c variant complexes and to J.B. Denault and T. Komiyama for helpful comments on the manuscript. We also thank R. Fuller and T. Komiyama for the eglin c variant libraries. This work was supported by the Canadian Institutes of Health Research (CIHR).

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