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Two engineered eglin c mutants potently and selectively inhibiting kexin or furin

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Abstract Eglin c with mutants L45R and D42R at the P_1 and P_4 positions has been reported to become a stable inhibitor toward the proprotein convertases (PC), furin and kexin, with a K_i of 2.3×10^{-8} and 1.3×10^{-10} M, respectively. The mutant was further engineered at the P_2'–P_4' positions to create a more potent and selective inhibitor for each enzyme. The residue Asp at P_1' which is crucial for stabilizing the conformation of eglin c remained unchanged. The eglin c mutants cloned into the vector pGEX-2T and expressed in *Escherichia coli* (DH5α) were purified to homogeneity, and their inhibitory activities toward the purified recombinant furin and kexin were examined. The results showed that (1) Leu47 at P_2' replaced with either a positively or negatively charged residue resulted in a decrease in inhibitory activities to both enzymes; (2) the replacement of Arg with Asp at P_3' was favorable for inhibiting furin with a K_i of 7.8×10^{-9} M, but not for inhibiting kexin; (3) the replacement of Tyr with Glu at P_4' increased the inhibitory activity to kexin with a K_i of 3×10^{-11} M, but was almost without any influence on furin inhibition. It was indicated that the inhibitory specificity of eglin c could be changed from inhibiting elastase to inhibiting PCs by site-directed mutation at the P positions, while the inhibitory selectivity to furin or kexin could be optimized by mutation at the P′ positions.
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Key words: Eglin c; Proprotein convertase; Furin; Kexin; Mutation

1. Introduction

The processing of precursor proteins via limited proteolysis at paired basic amino acids is an important and widely used cellular mechanism for the generation of biologically active proteins and peptides. The furin-like endoproteinases play broad and important roles in the proteolytic activation of not only most peptide hormones and neuropeptides but also many growth factors, receptors, adhesion molecules, serum proteins, plasma proteins and matrix metalloproteinases [1–5]. In addition to endogenous proteins, many pathogens of bacterial exotoxins and viral envelope glycoproteins also require these enzymes to be activated [6–11]. Kexin is the first discovered enzyme of this proprotein convertase (PC) family found in the yeast *Saccharomyces cerevisiae* [12,13]. Since then its mammalian counterparts have also been identified in mouse pituitary and human insulinoma as PC1/3, PC2 and others [14,15]. Among them, furin localized in the *trans*-Golgi network is ubiquitous as it is present in all tissues and cell lines examined [16]. Because of the homology of their catalytic domains with that of the bacterial serine protease, subtilisin, these enzymes are also called subtilisin-like PCs [17]. Mice with the furin gene knocked out died in the fetal period [18]. However, the furin-deficient cell lines were not infected by some viruses and exogenous pathogens [19]. In cellular and animal studies, the engineered variant of α_1-antitrypsin Portland (α_1-PDX) was used to block PC activity and to prevent the production of pathogenic viruses, bacterial toxin activation, and cancer metastasis [20].

Though small synthetic peptides could inhibit furin and kexin in vitro, their toxicity limits their possible therapeutic application [21]. It was reported that some protein inhibitors such as α_1-antitrypsin and ovomucoid were successfully engineered to become inhibitors for furin [22,23]. Our previous results showed that the eglin c variant with residues Leu and Pro at positions P_1 and P_4 replaced by a basic residue Arg (designated M1 in Table 1) exhibited a fairly strong inhibitory activity toward furin and kexin. If the P_2 residue Thr was also mutated to a basic residue (designated M2 in Table 1), the inhibitory activity was almost one order of magnitude higher. However, this inhibitor was unstable and cleaved slowly by the enzyme (temporary inhibition) [24,25]. Therefore, we chose M1 as the starting material to be further bioengineered at the P′ positions. According to molecular modeling there are distinct differences between the S′ subsites of furin and kexin, so the corresponding P′ residues of their selective inhibitors should also be different. As the P_1' residue Asp46 of eglin c plays an important role in maintaining the stable molecular conformation, this residue was unchanged [26]. The crystal structure of either furin or kexin has not been solved because of the instability of these enzymes. It would be desirable to find out a potent inhibitor and to obtain a stable inhibitor–enzyme complex to be crystallized. In this paper we report two engineered potent inhibitors selective for furin or kexin.

2. Materials and methods

2.1. Materials

All restriction enzymes, Taq DNA polymerase, Wizard PCR Preps DNA purification kit were from Promega. The GST-2T vector (glutathione *S*-transferase fusion protein expression system), thrombin, glutathione-Sepharose 4B and Chelating-Sepharose Fast Flow were from Amersham Pharmacia. The fluorogenic substrate pyrArg-Thr-Lys-Arg-MCA was from Bachem Bioscience. The pPICZ-C vector

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Table 1
Inhibition constants of the eglin variants to furin and kexin

Mutation		K_i		
		Furin (M)	Kexin (M)	
M1	RVTR	$2.3 \pm 0.08 \times 10^{-8}$	$1.28 \pm 0.21 \times 10^{-10}$	[24]
M2	RVKR	$1.6 \pm 0.27 \times 10^{-9}$	$3.8 \pm 0.16 \times 10^{-11}$	[24]
M3	RVTRD**E**RY	$9.2 \pm 0.13 \times 10^{-7}$	$3.16 \pm 0.31 \times 10^{-10}$	
M4	RVTRD**R**RY	$3.0 \pm 0.05 \times 10^{-7}$	$8.9 \pm 0.19 \times 10^{-8}$	
M5	RVTRDL**D**Y	$7.8 \pm 0.17 \times 10^{-9}$	$4.3 \pm 0.22 \times 10^{-9}$	
M6	RVTRDLR**R**	$4.7 \pm 0.06 \times 10^{-8}$	$2.67 \pm 0.24 \times 10^{-8}$	
M7	RVTRDLR**E**	$3.9 \pm 0.16 \times 10^{-8}$	$3.0 \pm 0.09 \times 10^{-11}$	
M8	RVTRDA**R**Y	$2.0 \pm 0.20 \times 10^{-8}$	$1.36 \pm 0.13 \times 10^{-10}$	

was from Invitrogen. Oligonucleotides were synthesized in the Shanghai Institute of Biochemistry and Cell Biology. The F-4010 fluorescence spectrophotometer was manufactured by Hitachi. The ultrafiltration system was from Millipore. The gene encoding prokexin was a gift from Dr. R.S. Fuller (University of Michigan Medical School). The purified furin was a gift from Dr. I. Lindberg (Louisiana State University).

2.2. Gene cloning of the eglin c variants

Site-directed mutagenesis of M1 was performed by polymerase chain reaction (PCR). The mutated gene was cloned through the flanking *Bam*HI and *Eco*RI restriction sites into the expression vector pGEX-2T provided by the GST kit. The nucleotide sequence of the mutant was verified by DNA sequencing. The primer pairs for the different mutants were as follows: RVTRD**E**RY (M3): 5′-CGTGACGAGCGTTACAA-3′, 5′-TTGTAACGCTCGTCACG-3′; RVTRD**R**RY (M4): 5′-CGTGACCGTCGTTACAA-3′, 5′-TTGTAACGACGGTCACG-3′; RVTRDL**D**Y (M5): 5′-CGTGACCTGGATTACAA-3′, 5′-TTGTAATCCAGGTCACG-3′; RVTRDLR**R** (M6): 5′-GACCTGCGTCGCAACCG-3′, 5′-CGGTTGCGACGCAGGTC-3′; RVTRDLR**E** (M7): 5′-GACCTGCGTGAGAACCG-3′, 5′-CGGTTCTCACGCAGGTC-3′; RVTRDA**R**Y (M8): 5′-CGTGACGCGCGTTACAA-3′, 5′-TTGTAACGCGCGTCACG-3′.

2.3. Expression and purification of eglin c variants

The expression vector was transformed into *Escherichia coli* strain DH5α as described [27]. Cells were grown in LB medium containing 2% (w/v) glucose and 100 μg/ml ampicillin until OD_{600} reached 0.8. The expression of the fusion protein GST-eglin c mutant was then induced with isopropylthiogalactoside. The harvested cells were treated with ultrasonic wave. The fusion protein was purified by affinity chromatography with a glutathione-Sepharose 4B column. The eluted fraction was hydrolyzed by thrombin. The eglin c variant was purified by reverse phase high performance liquid chromatography (HPLC) with gradient elution from 100% buffer A (0.1% trifluoroacetic acid) to 70% buffer B (70% acetonitrile with 0.1% trifluoroacetic acid) in 50 min. The purified eglin c mutant was lyophilized for inhibitory activity assay.

2.4. Gene cloning and expression of the secreted soluble kexin

The kexin gene with the C-terminal 201 residues deleted was cloned into the pPICZ-C vector using the *Eco*RI and *Xho*I restriction sites. There were 6 His (for affinity chromatography) and a c-myc sequence at the C-terminus. The transformation of the plasmid into *Pichia pastoris* and the selection of the Mut^+ clones were performed as described [28]. After induction by methanol for 72 h, the media were pooled and ultra-filtered using a 10 kDa cutoff membrane in a 50 ml stirred cell (Millipore) and washed with 20 mM phosphate buffer, pH 7.0, containing 1 mM $CaCl_2$ and 1 M NaCl. The concentrated sample was applied to a Chelating-Sepharose Fast Flow affinity column and eluted with 20 mM phosphate buffer, pH 7.0, containing 1 mM $CaCl_2$ and 1 M imidazole. The eluted fraction was concentrated using Centricon 10 (Amicon) and dialyzed against 20 mM HEPES buffer, pH 7.5, containing 1 mM $CaCl_2$ and 10% glycerol, and stored at −70°C.

2.5. Enzyme activity assays

The enzyme activity of furin and kexin was measured at 37°C in a volume of 1 ml using 1 μM or 2 μM fluorogenic MCA substrate pyrArg-Thr-Lys-Arg-MCA in 100 mM HEPES buffer, pH 7.5, 1 mM $CaCl_2$, 0.5% Triton X-100 and 1 mM β-mercaptoethanol. For each assay an equivalent amount of enzyme was added to release 15 nM/min amino-4-methylcoumarin in the period of 2 min of enzyme reaction. For determining the inhibitory activity, a fixed amount of enzyme was first incubated with different amounts of the inhibitor at 37°C for 5 min, the residual enzyme activity was then measured. The incubation time required for reaching the equilibrium between enzyme and inhibitor was estimated to be less than 5 min as all initial velocities were the same from 1 to 30 min incubation. The excitation and emission wavelengths were 370 nm and 460 nm, respectively.

2.6. Measurement of kinetic parameter, K_i

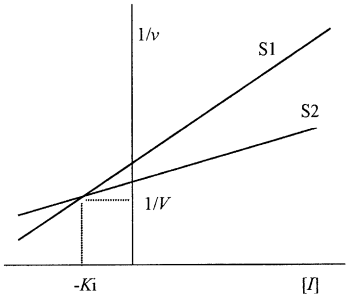
The K_i values for furin or kexin were measured by Dixon's plot ($1/V$ against I) using different concentrations of substrate [29]. Data from three measurements were averaged and graphically analyzed with the following equation to obtain the equilibrium inhibition constant, K_i. The equilibrium equation could be simplified as follows:

$$\frac{v}{V_{max}} = \frac{[S]}{K_m + [S] + \frac{K_m}{K_i}[I]}$$

$$v = \frac{1}{a[I] + b} \text{ or } \frac{1}{v} = a[I] + b \quad (1)$$

$$a = \frac{K_m}{K_i \cdot V_{max}[S]} \quad (2)$$

$$b = \frac{K_m + [S]}{V_{max}[S]} \quad (3)$$



K_m is the Michaelis constant, the substrate K_m was 500 μM and 2.7 nM for furin and kexin respectively, $[S]$ is the substrate concentration, V_{max} is the initial uninhibited rate. The abscissa of the intersection point of lines is $-K_i$.

3. Results

3.1. Expression and purification of kexin

The gene encoding kexin with 201 residues deleted from the C-terminal part was cloned into the pPICZ-C vector and expressed in *P. pastoris*. After ultrafiltration with a 10 kDa cutoff membrane, the expressed recombinant enzyme was purified by affinity chromatography with a Chelating-Sepharose Fast Flow column (Fig. 1). Because of the low capacity of the column, the unbound kexin could also be found in the breakthrough. The purified kexin was stored at −70°C.

3.2. Expression and purification of eglin c variants

The synthetic gene encoding eglin c was constructed by

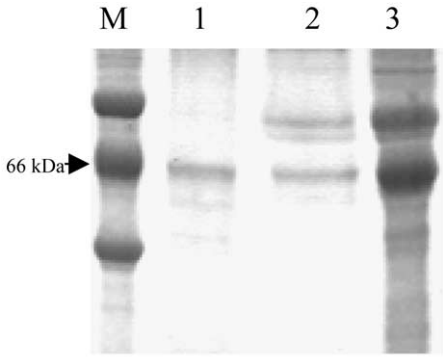


Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the purified kexin. Lane 1: fraction eluted from the activated Chelating-Sepharose Fast Flow column with 20 mM phosphate buffer, pH 7.0, containing 1 mM $CaCl_2$ and 1 M imidazole; lane 2: breakthrough from the column; lane 3: starting material concentrated by ultrafiltration with a 10 kDa membrane in a 50 ml stirred cell.

using preferential codon usage for *E. coli* [21,30]. The GST fusion protein expression system was used for all eglin c variants with a yield of around 5 mg/l. The fusion protein was purified by one-step affinity chromatography on the glutathione-Sepharose 4B column, and cleaved by thrombin (Leu-Val-Pro-Arg↓-Gly-Ser, ↓ indicates the site of cleavage) upstream of the N-terminus of eglin c, resulting in a recombinant Gly-Ser-eglin c. The cleaved product was purified by HPLC using C18 column (Figs. 2 and 3).

3.3. *Effect of P′ mutation of M1 on the inhibitory activity and selectivity for furin and kexin*

Considering the difference of S′ subsites between furin and kexin, the residues of eglin c mutant M1 were further engineered (L47E, L47R, L47A, R48D, Y49R and Y49E) at positions P′ to explore whether these P′ mutations would influence its inhibitory activity and selectivity for furin and kexin.

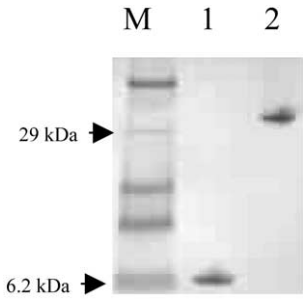


Fig. 2. SDS–PAGE analysis of the purified eglin c variant. Lane 1: the purified eglin c variant cleaved with thrombin from the fusion protein; lane 2: the fusion protein purified with glutathione-Sepharose 4B column.

The determined inhibition constants K_i of the mutants are listed in Table 1. The L47E mutation (M3) at P_2' resulted in a 40-fold decrease in inhibitory activity for furin, and a 2.5-fold decrease for kexin. The L47R mutation (M4) at the same position had a small effect on furin inhibition (13-fold decrease) but a dramatic effect (700-fold decrease) on kexin inhibition, indicating that the neutral residue at position P_2' of eglin c was needed to interact with the enzyme. Both mutations with either an acid or basic residue gave a negative effect. The acidic residue mutation showed more influence on furin inhibition than on kexin inhibition, while the influence of the basic residue mutation was reversed. In order to exclude the possible influence of the steric effect of the hydrophobic residue Leu on K_i values, this position was further mutated with the small neutral residue Ala. The result showed that the determined K_is of the L47A mutation (M8) were within errors, indicating that the P_2' can be replaced by another neutral residue, but not by charged residues. The P_3' R48D mutation (M5) increased three-fold the binding affinity of M1 for furin, but decreased it 33-fold for kexin. Thus, M5 exhibited a high degree of selectivity for furin, its inhibitory

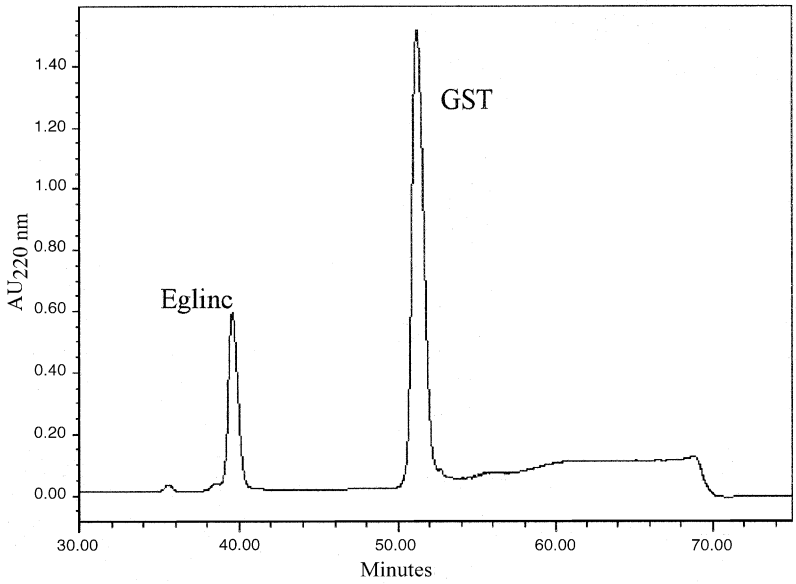


Fig. 3. HPLC profile of the purified fusion protein eglin-GST after enzymatic cleavage with thrombin.

activity was close to that of the suicide inhibitor M2 with a K_i of 7.8×10^{-9} M. The P_4' Y49R mutation (M6) decreased the binding affinity of M1 for kexin about 200-fold. In contrast, the Y49E mutation (M7) increased the affinity about four-fold. Thus, the inhibitory activity of M7 approached that of the suicide inhibitor M2 and became a potent and selective inhibitor of kexin with a K_i of 3×10^{-11} M. However, neither Y49R nor Y49E mutations at position P4′ had an obvious influence on the inhibitory activity of M1 to furin.

4. Discussion

The 70 residue eglin c, isolated from the leech *Hirudo medicinalis*, belongs to the potato I inhibitor family and inhibits strongly human leukocyte elastase, cathepsin G, α-chymotrypsin, and subtilisin [31–33]. Because of its low molecule weight, stable conformation and flexible reactive site loop, eglin c has been successfully engineered at the P positions to become an inhibitor of the subtilisin family enzymes. Though a very potent inhibitor with a K_i of 10^{-9} and 10^{-11} M to furin and kexin respectively was generated, the mutant was unstable and slowly cleaved by the corresponding enzyme [24,25]. Thus, it is necessary to make further mutations at the P′ positions to create a potent, selective and stable inhibitor for furin and kexin. It was demonstrated that in eglin c the acid residue Asp46 at position P_1' formed an electrostatic interaction with the basic residue Arg51, playing a role like a disulfide bond in the Kunitz family inhibitors [26]. Therefore, Asp46 should remain unchanged. The fact that mutant M5 (R48D) showed a higher inhibitory activity to furin but was unfavorable for kexin inhibition could be explained by the molecular modeling of the furin–eglin complex [34]. The P_3' residue Asp48 of eglin c is close to the S_3' residue possibly either Lys242 or His257 of furin, forming an electrostatic interaction. In kexin this acid residue Asp48 is neighboring to Asp80 of the enzyme, so the electrostatic repulsion between these residues is unfavorable for forming an enzyme–inhibitor complex. In M7, the P_4' residue Glu49 could also form an electrostatic interaction with His250 of kexin. Thus, M5 and M7 turned out to be potent and selective inhibitors for furin and kexin, respectively. Their inhibitory activities are almost equivalent to that of the suicide inhibitor M2 mutated only at the P positions [24,25]. However, unlike M2, they are stable and not cleaved by their corresponding enzymes.

Naturally occurring inhibitors for PCs have not been found yet. Until now several protein inhibitors of PCs have been reported such as the engineered ovomucoid third domain inhibitor, α_1-PDX, and eglin c variants [22–25,35]. The effective inhibition of PCs by α_1-PDX in vivo has been confirmed both by expressing the α_1-PDX gene in the target cells and by adding the purified α_1-PDX to tissue culture media [23,36,37]. However, the large size (∼400 amino acids) and relative instability of the serpin family inhibitor may hamper the potential therapeutic application. The eglin variant M5 for furin inhibition reported in this paper may have more advantages because of its low molecule weight and stability.

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