Rational redesign of inhibitors of furin/kexin processing proteases by electrostatic mutations[1]

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ABSTRACT

AIM: To model the three-dimensional structure and investigate the interaction mechanism of the proprotein convertase furin/kexin and their inhibitors (eglin c mutants). **METHODS:** The three-dimensional complex structures of furin/kexin with its inhibitors, eglin c mutants, were generated by modeller program using the newly published X-ray crystallographical structures of mouse furin and yeast kexin as templates. The electrostatic interaction energy of each complex was calculated and the results were compared with the experimentally determined inhibition constants to find the correlation between them. **RESULTS:** High quality models of furin/kexin-eglin c mutants were obtained and used for calculation of the electrostatic interaction energies between the proteases and their inhibitors. The calculated electrostatic energies of interaction showed a linear correlation to the experimental inhibition constants. **CONCLUSION:** The modeled structures give good explanations of the specificity of eglin c mutants to furin/kexin. The electrostatic interactions play important roles in inhibitory activity of eglin c mutants to furin/kexin. The results presented here provided quantitative structural and functional information concerning the role of the charge-charge interactions in the binding of furin/kexin and their inhibitors.

INTRODUCTION

Serine proteinase furin and kexin are members of a large family of eukaryotic proprotein processing proteases. Because the catalytic domains of the furin/kexin processing proteases are homologous to that of the bacterial serine protease, subtilisin, these enzymes are also called subtilisin-like proprotein convertases. These enzymes activate a large number of proprotein substrates by cleaving at paired basic residue sites. The consensus cleavage site is positioned after the carboxyl-terminal arginine residue in the sequence -R-X-K/R-R$^{\downarrow}$- for furin and -K/R-R$^{\downarrow}$- for kexin, where the arrow ↓ identifies the cleavage site. Their substrates include peptide hormones, neuropeptides, growth factors, cell-surface receptors, adhesion molecules, serum proteins, plasma proteins and matrix metalloproteinases. In addition, these enzymes can also activate many pathogens of bacterial exotoxins and viral envelope glycoproteins[1]. The furin-like endoprotease plays broad and important roles in embryogenesis and in diseases ranging from anthrax, bird flu, Ebola fever to cancer and dementia[2]. Thus it is of great importance to develop efficient furin inhibitors.

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Though efforts have been made to find naturally occurring protein inhibitor of the furin-like enzyme[3], native inhibitors of furin have not been found yet. Some small molecules or peptides such as peptidyl chloromethyl ketones and polyarginines may inhibit furin/kexin efficiently. However, concerns about toxicity and turnover of inhibitors limit the possible therapeutic applications of these molecules. Several protein inhibitors of furin/kexin have been described such as the engineered ovomucoid third domain, α1-antitrypsin portland (α1-PDX), kexstatin, and eglin c variants[2,4,5]. The 70-residue protein eglin c from the leech *Hirudo medicinalis* is a potent protein inhibitor of many serine proteases, such as chymotrypsin and subtilisins. The binding loop of eglin c was reconstructed by charge mutation to change its inhibition specificity against furin and kexin. It is reported that the eglin c mutant with residues Leu and Pro at positions P_1 and P_4 replaced by basic residue Arg exhibited a fairly strong inhibitory activity toward furin and kexin (substrate residues are designated as P_4-P_3-P_2-P_1*P_1'-P_2'-P_3'-P_4' with the scissile peptide bond between P_1 and P_1' indicated by "*"; the corresponding subsites that constitute the topography of the active site cleft in each enzyme are designated accordingly as S_4-S_3-S_2-S_1-S_1'-S_2'-S_3'-S_4'). If the P_2 residue Thr44 was also mutated to a basic residue Lys, the inhibitory activity was almost one order of magnitude higher. However, this eglin mutant was unstable and was slowly cleaved by the enzyme (temporary inhibition)[4,6]. The stable eglin c-RVTR45 mutant was further engineered at P' positions. Two potent inhibitors, R48D and Y49E, which were selective for furin and kexin respectively, were obtained (Tab 1)[1,7].

Siezen *et al*[8,9] and Ye *et al*[7] had modeled the structures of furin and kexin and attempted to explain the molecular basis of specificity of the two enzymes. However, limited by the structure templates they used, the interaction mechanism of furin/kexin with eglin c mutants is still not accurately determined. The recently disclosed high resolution crystal structures of the ligand binding domain and P domain of mouse furin and yeast kexin with their inhibitors, may represent a breakthrough in understanding the inhibition mechanism of these two proteases and the rational design of novel furin/kexin inhibitors[10-12]. Here we modeled a set of complex structures of eglin c mutants with furin/kexin using the newly solved crystal structures of mouse furin and yeast kexin as templates. This work will give insight into the molecular mechanism of the selectivity and specificity of eglin c mutants to the proprotein convertases furin and kexin, and thus can be used in the rational design of antiviral and antibacterial drugs.

MATERIALS AND METHODS

Structure template The 2.6 Å resolution crystal structure of the decanoyl-Arg-Val-Lys-Arg-chloromethylketone inhibited mouse furin (PDB code 1P8J) was used as structure template of human furin. The structure of kexin was directly taken from its complex with inhibitor Ac-Ala-Lys-BoroArg at 2.4 Å resolution (PDB code 1OT5). Eglin c-RVTR45 mutant was modeled using two complex structures: eglin c-L45R mutant in complex with subtilisin at 2.1 Å resolution (PDB code 1SBN) and eglin c in complex with thermitase at 2.0 Å resolution (PDB code 3TEC).

Modeling complex structures Since the sequence of human furin is highly homologous to that of mouse, they can be easily aligned. The inhibitor fragments RVKR (in 1P8J) and AKR (in 1OT5) were manually aligned to the corresponding P positions preceding the cleavage site of eglin c in 1SBN and 3TEC. The complex structures of furin/kexin with eglin c-RVTR45 were modeled using the well known homology modeling program MODELLER[13] The structures were evaluated by Procheck[14]. The structures of furin/kexin in complex with other eglin c mutants were constructed by SCEO method as described previously[15,16].

Correlation of electrostatic interaction energies and inhibition constants According to classic theory the association of enzyme and its inhibitor is described as:

$$E+I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI$$

where k_{on} and k_{off} are the rate constants of association and dissociation. It had been demonstrated that for association of two proteins, only charged residues alter k_{on}. The change in k_{on} shows a linear relation to the change in electrostatic energy of interaction between the two proteins, while k_{off} is relatively insensitive to the electrostatic potential[17,18]. Increasing long range electrostatic attraction through electrostatical mutations increases specifically k_{on}, but does not affect k_{off}, with the increase being proportional to the increase in Debye-Hückel energy of interaction (ΔU) of the final complex relative to the unbound proteins[19-21]:

$$\ln k_{on}=\ln k^0_{on}-\beta\Delta U/RT$$

where k_{on}^0 is the basal rate of association in the absence of electrostatic forces or the charge independent part of the association rate, β is a constant related to ionic strength.

The inhibitor constant (K_i) is a dissociative equilibrium constant of EI and can be expressed as a function of the rates of association and dissociation: $K_i = k_{off}/k_{on}$. Therefore the change in K_i should also show an approximate linear relation to the change in electrostatic energy of interaction:

$$\ln K_i = \ln k_{off} - \ln k_{on}^0 + \beta \Delta U/RT$$

Based on this principle, the electrostatic interaction energies of furin/kexin with eglin c mutants were calculated using the method described by Selzer *et al*[19,21]. The Debye-Hückel energy of a protein is calculated using:

$$U = \frac{1}{2} \sum_{i,j} \frac{q_i q_j}{4\pi\varepsilon_0 \varepsilon r_{ij}} \frac{e^{-\kappa(r_{ij}-a)}}{1+\kappa a}$$

where *i* and *j* are the charged atoms in the proteins, ε is the dielectric constants (set to 80) and κ is the Debye-Hückel screening parameter that relates to the ionic strength of the solution. A κ value of 0.8, which is corresponding to the experimental condition, was used in the calculation. The electrostatic energy of interaction between two proteins was calculated from the difference between the sum of the electrostatic energy of the two individual proteins and the electrostatic energy of the complex:

$$\Delta U = U_{complex} - U_{protein1} - U_{protein2}$$

The modeled complex structures were used in the calculation and coordinates for the individual proteins were taken from the structure of the complex.

RESULTS AND DISCUSSION

Interaction of furin/kexin with eglin c mutants The refined models have good stereochemistry: 90.5 %, 9.0 %, and 0.4 % of main chain phi/psi angles of furin-RVTR eglin c complex and 89.9 %, 9.4 %, and 0.4 % that of kexin-RVTR eglin c complex are in core, allowed and generously allowed regions as evaluated using Procheck. The root mean square deviation (RMSD) of C_α traces between the refined furin/kexin models and the corresponding crystal structures of furin/kexin is 0.54 Å and 0.25 Å, respectively, which is in the limit of the crystallographic resolution (<1.0 Å). Structural analysis shows that the inhibitor's P_3, P_2, and P_1 residues form a twisted antiparallel β-sheet with Ser146, Trp147, and Gly148 segment of furin (we use the residue numbering scheme taken by Siezen and Ye *et al*[7-9] throughout this paper) and Ser272, Trp273, and Gly274 of kexin. There are many charged residues present at the interaction interface of furin/kexin and eglin c mutant. The conserved antiparallel β-sheet and charge-charge interactions may be important for the stability and specificity of furin/kexin-inhibitor binding.

P specificity The P_1 residue Arg45 is important for binding of eglin c mutant toward furin/kexin. When the two complexes structures (furin-eglin c-RVTR and kexin-eglin c-RVTR) were superimposed, it showed that the interactions of furin and kexin are almost identical at P_1 site. In the case of furin, the terminal guanidinium group of R45 of eglin c mutant forms a perfect electrostatic and hydrogen bonding network with the carboxylate oxygen of D151 and D199 and the carbonyl oxygen of A185 and P149[10] (Fig 1A). While in kexin, the arrangement of amino acid residues contributing to the S_1 binding pocket is almost the same as those in furin[6] (Fig 1B). The aliphatic portion of the arginine side chain has very little interaction with the S_1 pocket in both furin and kexin. These interaction patterns explain the reason why both furin and kexin have a stringent selectivity for arginine at the P_1 site and substitution of R45 with any other residue will result in loss of some interactions and dramatic reduction in the inhibition efficiency of eglin c.

The P_2 site is surrounded by acid residues D175, D176, D210, and D211 in kexin and D46, D47, D84, and D121 in furin. So a basic residue with positive charge can make strong electrostatic interactions with these residues and is most favorable residue at this site which is consistent with the experimental mutation studies.

Both furin and kexin are lack of preference at the P_3 site. But basic P_3 residues are frequently present in *in vivo* substrates of furin[10], which could make favorable contacts with E150 or D151. While kexin has shown no positive selectivity exists at P_3, this may because that the geometry of this site can not accommodate a long basic residue.

It is interesting to compare the structural determinants of selectivity of P_4 site between furin and kexin. Kexin has a dual selectivity for aliphatic and basic residues at this position, while replacement of arginine at this site in biological substrates essentially abolish activity in furin[5]. Structural analysis shows that the P_4

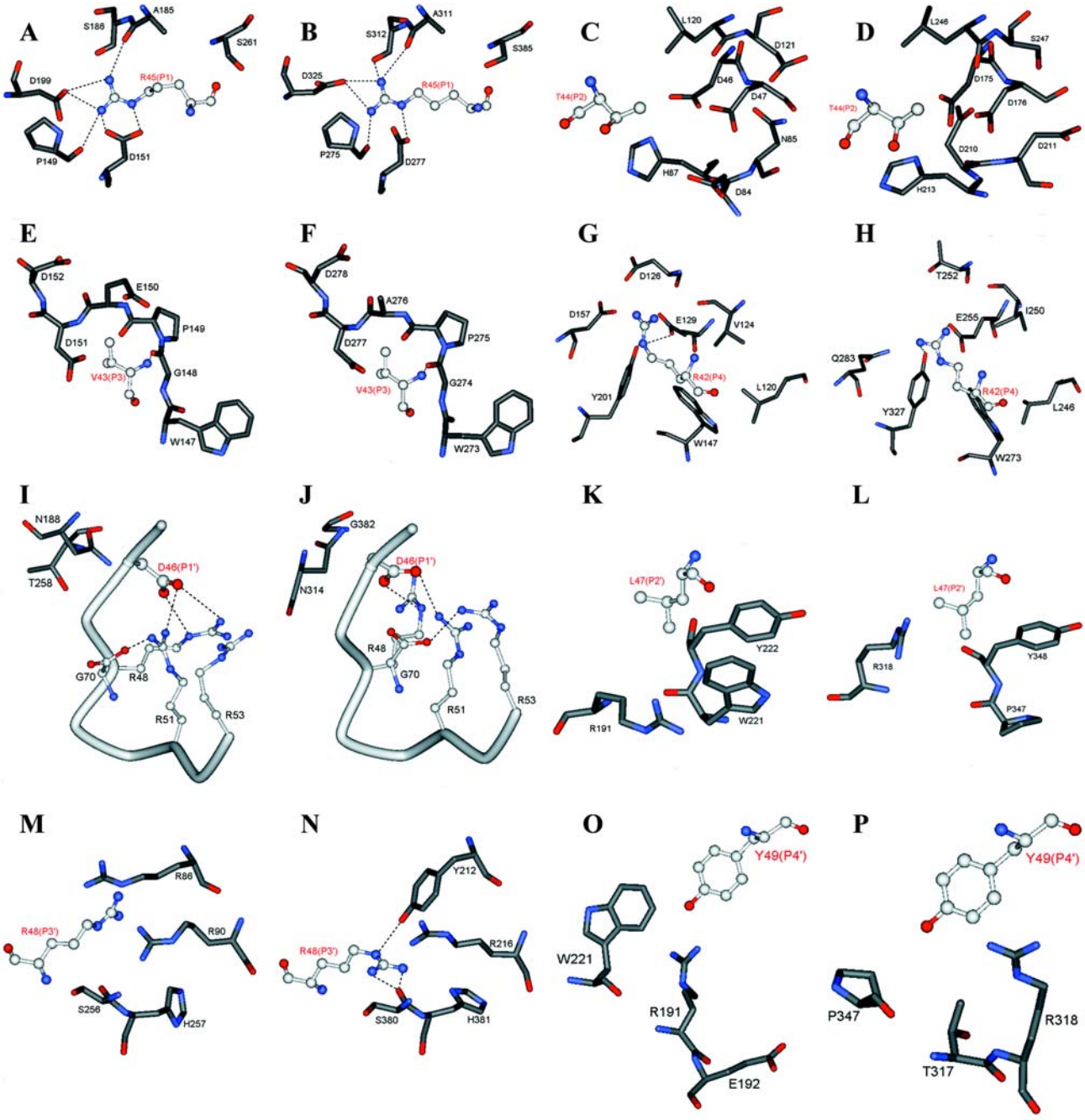

Fig 1. Close up view of binding site of eglin c-RVTR mutant to furin/kexin illustrating the arrangement of the P and P' subsites. Those amino acids making contacts with the inhibitor were shown. A, C, E, G, I, K, M, and O: Interaction of P_4-P_4' subsites of eglin c mutant with furin. B, D, F, H, J, L, N, and P: Interaction of P_4-P_4' subsites of eglin c mutant with kexin. The binding pockets were shown as stick model and subsites of inhibitor as ball-and-stick.

residue R42 is packed against W273, Q283, Y327 with E255 at the bottom of the S_4 binding pocket of kexin and this S_4 pocket is predetermined by the enzyme and is independent of the occupancy of a P_4 residue[12]. Whereas the S_4 pocket of furin is comprised of W147 and Y201 with D126, E129, and D157 positioned at the bottom of the pocket to provide the electrostatic interaction with basic residue R42. The overall arrangement of these residues is very similar in the two enzymes while still exists some differences. The spatial arrangement of residues in the S_4 pocket of kexin is looser than that of furin and the positions of kexin corresponding to furin D126 and D157 are not acid residues but T252 and Q283. A possible reason for the P_4

arginine preference of furin may be that this arginine makes an electrostatic network with three acid residues in the S_4 pocket and form an elaborate interaction at this site, which is similar to that observed in binding of P_1 arginine. Substitution for arginine will destroy the electrostatic network and the repulsion forces between three negatively charged acid residues may change the conformation of the S_4 pocket, thus result in decreasing affinity in the binding of eglin c mutant.

P′ Specificity The acid residue Asp46 at site P_1' is crucial for stabilizing the conformation of eglin c. The binding loop of eglin c is predominantly stabilized by a network of electrostatic and hydrogen bonding interactions, involving Asp46, three arginine residues (Arg48, Arg51, and Arg53) and Gly70 of this molecule. It is reported that though C-terminal residue Gly70 is not involved in the main binding interface, it plays an important role in supporting the binding loop of eglin c by making hydrogen bond with Arg51 or Arg 53, which in turn interacts with the P_1′ Asp46. Compromising the integrity of the interaction between Asp46 and Arg51 invariably results in weak binding or temporary inhibition to target enzymes. If Asp46 is mutated to a serine residue, the internal rigidity of the binding loop is significantly weakened due to the loss or destabilization of the internal hydrogen bond of the P_1′ residue[22,23]. So this residue is kept unchanged in eglin c mutants.

Although eglin c interact with furin/kexin primarily by electrostatic or hydrogen bonding interactions, there are still hydrophobic effects involved. The Leu residue at P_2′ site forms a small hydrophobic interaction with Trp221 and Tyr222 in furin and Pro347 and Tyr348 in kexin. The P_4′ residue Tyr49 also participates in the hydrophobic interaction. But as analyzed below, these hydrophobic interactions may have only minor effects on the binding of furin/kexin with eglin c mutants. Replacement of Leu47 with charge residues results in great decline in inhibition efficiency, which may be caused by unfavorable desolvation and electrostatic effects of charged residues. The great decrease of inhibition constant of kexin-eglin c L47R mutant may be attributed to the repulsion force between R47 of eglin mutant and R318 of kexin. On the contrary, the L47E mutant has a relatively short side chain at this site and can only make weak effect on the inhibition constant. In the case of furin, the interaction mode at this site is similar to that of kexin.

The P_3′ residue Arg48 of eglin c forms hydrogen bonds with the main-chain carbonyl of Ser380 and the hydroxyl of Tyr212 in kexin. Replacement of Arg48 with an acid residue Asp causes loss of these hydrogen bonds, in consistent with the reduced inhibition efficiency of this mutant. However, in furin, the eglin c-R48D mutation may form favorable electrostatic interactions with Arg86 and Arg90 of furin, resulting in an increase in inhibition efficiency (Fig 2A).

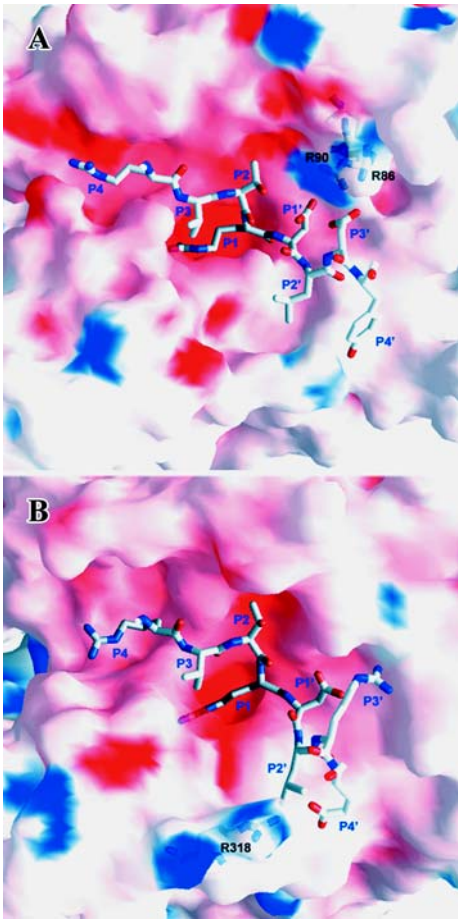

Fig 2. Different selectivity of eglin c mutants to furin and kexin. The P_4-P_4′ sites of inhibitor (stick model) were shown in front of the surface of the binding cleft of enzyme, colored according to its electrostatic surface potential. A: Interaction model of the eglin c-RVTRDLDY mutant with furin. B: Interaction model of the eglin c-RVTRDLRE mutant with kexin.

When complexed with furin, the P_4′ residue Tyr49 of eglin c is largely exposed to the solvent; substituting either Arg or Glu for Tyr at this site causes no significant change in inhibition constants. In kexin complex, replacing Tyr49 with Arg will cause electrostatic repulsion with Arg318, which results in decrease of binding affinity. On the contrary, the Y49E mutation increases the affinity through either electrostatic or hydrogen

bonding interactions with the basic residue (Fig 2B), making this mutant a potent and selective inhibitor to kexin.

Interaction mechanism of furin/kexin with eglin c mutants From the analysis above, we can see that the electrostatic interaction play an important role in the binding of eglin c mutants to furin/kexin. To further investigate the effects of electrostatic interactions on the inhibition efficiency of eglin c mutants against furin/kexin, the electrostatic binding energy of each complex in Tab 1 were calculated using method described above. The calculated electrostatic interaction energies of each complex were listed in the same table, and the data were linearly correlated to the corresponding inhibition constants and good correlations were found. Fig 3 shows the plot of ln K_i versus ΔU/RT for furin and kexin, and the correlation coefficient is 0.96 for furin and 0.94 for kexin. The linear correlation suggests that the change in the inhibition potency of furin/kexin by eglin c mutants is dominated by electrostatic interactions.

Tab 1. Inhibition constant values (K_i) of the eglin c variants toward furin and kexin and the calculated electrostatic energy of interaction.

Mutation	Furin		Kexin	
	K_i (mol/L)	ΔU (kcal/mol)	K_i (mol/L)	ΔU (kcal/mol)
PVTR-DLRY$_{49}$*	-	-0.78	7.4×10^{-9}	-1.27
PVKR-DLRY$_{49}$*	-	-2.00	3.9×10^{-10}	-3.07
RVTR-DLRY$_{49}$*	1.9×10^{-8}	-3.02	2.8×10^{-10}	-2.42
RVKR-DLRY$_{49}$*	1.6×10^{-9}	-4.77	3.8×10^{-11}	-4.29
RVTR-DERY$_{49}$	9.2×10^{-7}	-1.44	3.2×10^{-10}	-2.94
RVTR-DRRY$_{49}$	3.0×10^{-7}	-2.43	8.9×10^{-8}	-0.88
RVTR-DLDY$_{49}$	7.8×10^{-9}	-4.45	$4.3\text{x}10^{-9}$	-2.68
RVTR-DLRR$_{49}$	4.7×10^{-8}	-3.40	2.7×10^{-8}	-1.31
RVTR-DLRE$_{49}$	3.9×10^{-8}	-3.19	3.0×10^{-11}	-3.94

* The K_i values were taken from reference [6]. The K_i values of all other mutants were taken from reference [1].

As for the individual mutant, the P_2' site is located near the negatively charged patch of furin/kexin and positively charged residue R191 of furin or R318 of kexin. Substituting a basic residue for Val47 of eglin c leading to electrostatic repulsion to adjacent Arg residue of enzyme. While mutating Val47 to an acid residue does not result in more favorable electrostatic attractions but unfavorable desolvation effects and repulsion interactions with the binding interface of the enzyme. That is the reason why charge mutation at P_2' site diminishes the inhibitory activity of eglin c. On the other hand, mutating Arg48 of eglin c to an acid residue can relieves the local electrostatic repulsion and form favorable attraction with basic surface residues of furin, and in kexin mutating Tyr49 to an acid residue has similar effects. These effects result in faster association of eglin c mutants with furin/kexin, thus increasing the inhibition activity toward their corresponding enzymes.

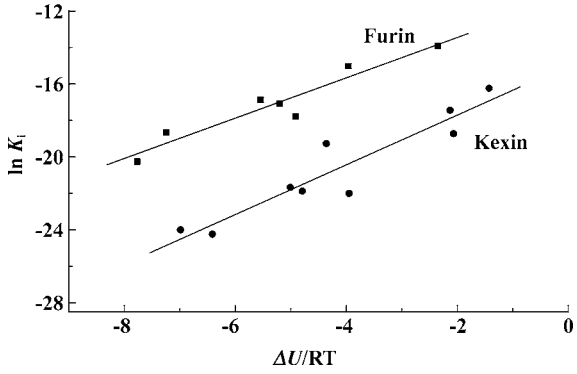

Fig 3. Plot of ln K_i versus the electrostatic energy of interaction of furin and kexin with various eglin c mutants.

In conclusion, the modeled complex structures are of high quality and give good explanations of the experimental specificity of eglin c mutants to furin/kexin. Good correlations were found between the calculated electrostatic interaction energies and the corresponding inhibition constants. The electrostatic interaction play an important role in inhibitory activity and determining substrate specificity of furin/kexin. The results presented here provided quantitatively structural and functional information concerning the role of the charge-charge interactions in the binding of furin/kexin to their inhibitors, which might offer a strategy in the rational design of novel specific furin/kexin inhibitors.

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REFERENCES

1 Liu ZX, Fei H, Chi CW. Two engineered eglin c mutants potently and selectively inhibiting kexin or furin. FEBS Lett 2004; 556: 116-20.

2 Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. Nat Rev Mol Cell Biol 2002; 3:

753-66.
3 Fei H, Li Y, Wang LX, Luo MJ, Ling MH, Chi CW. Nonhistone protein purified from porcine kidney acts as a suicide substrate inhibitor on furin-like enzyme. Acta Pharmacol Sin 2000; 21: 265-70.
4 Komiyama T, Fuller RS. Engineered eglin c variants inhibit yeast and human proprotein processing proteases, Kex2 and furin. Biochemistry 2000; 39: 15156-65.
5 Rockwell NC, Krysan DJ, Komiyama T, Fuller RS. Precursor processing by kex2/furin proteases. Chem Rev 2002; 102: 4525-48.
6 Fei H, Luo MJ, Ye YZ, Ding DF, Chi CW. The inhibitory activities of recombinant eglin C mutants on kexin and furin, using site-directed mutagenesis and molecular modeling. Acta Biochim Biophys Sin 2001; 33: 591-9.
7 Ye YZ, Fei H, Ding DF. Rational redesign of inhibitors of furin/kexin processing proteases. Acta Biochim Biophys Sin 2001; 33: 504-12.
8 Siezen RJ, Leunissen JA. Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci 1997; 6: 501-23.
9 Siezen RJ, Creemers JW, Van de Ven WJ. Homology modelling of the catalytic domain of human furin. A model for the eukaryotic subtilisin-like proprotein convertases. Eur J Biochem 1994; 222: 255-66.
10 Henrich S, Cameron A, Bourenkov GP, Kiefersauer R, Huber R, Lindberg I, *et al*. The crystal structure of the proprotein processing proteinase furin explains its stringent specificity. Nat Struct Biol 2003; 10: 520-6.
11 Holyoak T, Wilson MA, Fenn TD, Kettner CA, Petsko GA, Fuller RS, *et al*. 2.4 Å resolution crystal structure of the prototypical hormone-processing protease Kex2 in complex with an Ala-Lys-Arg boronic acid inhibitor. Biochemistry 2003; 42: 6709-18.
12 Holyoak T, Kettner CA, Petsko GA, Fuller RS, Ringe D. Structural basis for differences in substrate selectivity in Kex2 and furin protein convertases. Biochemistry 2004; 43: 2412-21.
13 Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 1993; 234: 779-815.
14 Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Crystallogr 1993; 26: 283-91.
15 Ye YZ, Tang HX, Ding DF. Engineering novel functional proteins grafting active sites into natural scaffolds. Acta Biochim Biophys Sin 1999; 31: 303-08.
16 Ye YZ, Tang HX, Ding DF. Design of protein cores by screening combinatorial sequence library. Acta Biochim Biophys Sin 1998; 30: 525-39.
17 Zhou HX. Theory and simulation of the influence of diffusion in enzyme-catalyzed reactions. J Phys Chem. B 1997; 101: 6642-51.
18 Zhou HX, Wong KY, Vijayakumar M. Design of fast enzymes by optimizing interaction potential in active site. Proc Natl Acad Sci USA 1997; 94: 12372-7.
19 Selzer T, Albeck S, Schreiber G. Rational design of faster associating and tighter binding protein complexes. Nat Struct Biol 2000; 7: 537-41.
20 Selzer T, Schreiber G. New insights into the mechanism of protein-protein association. Proteins 2001; 45: 190-8.
21 Selzer T, Schreiber G. Predicting the rate enhancement of protein complex formation from the electrostatic energy of interaction. J Mol Biol 1999; 287: 409-19.
22 Heinz DW, Hyberts SG, Peng JW, Priestle JP, Wagner G, Grutter MG. Changing the inhibitory specificity and function of the proteinase inhibitor eglin c by site-directed mutagenesis: functional and structural investigation. Biochemistry 1992; 31: 8755-66.
23 Lu WY, Starovasnik MA, Dwyer JJ, Kossiakoff AA, Kent SB, Lu W. Deciphering the role of the electrostatic interactions involving Gly70 in eglin C by total chemical protein synthesis. Biochemistry 2000; 39: 3575-84.