Identification of furin pro-region determinants involved in folding and activation

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The pro-region of the subtilisin-like convertase furin acts early in the biosynthetic pathway as an intramolecular chaperone to enable proper folding of the zymogen, and later on as an inhibitor to constrain the activity of the enzyme until it reaches the *trans*-Golgi network. To identify residues that are important for pro-region function, we initially identified amino acids that are conserved among the pro-regions of various mammalian convertases. Site-directed mutagenesis of 17 selected amino acids within the 89-residue pro-region and biosynthetic labelling revealed that I60A-furin and H66A-furin were rapidly degraded in a proteasome-dependent manner, while W34A-furin and F67A-furin did not show any autocatalytic activation. Intriguingly, the latter mutants proteolytically cleaved pro-von Willebrand factor

precursor to the mature polypeptide, suggesting that the mutations permitted proper folding, but did not allow the pro-region to exercise its role in inhibiting the enzyme. Homology modelling of furin's pro-region revealed that residues Ile-60 and His-66 might be crucial in forming the binding interface with the catalytic domain, while residues Trp-34 and Phe-67 might be involved in maintaining a hydrophobic core within the pro-region itself. These results provide structural insights into the dual role of furin's pro-region.

Key words: enzymic activation, folding, furin, pro-region, proteolytic processing, zymogen.

INTRODUCTION

Endopeptidases are often synthesized as inactive precursors or zymogens, which are converted into active enzymes within a specific subcellular compartment or in the extracellular environment [1–3]. This mechanism prevents premature proteolysis and targets enzymic activities to the compartment where their function is required. In many cases, activation occurs by the removal of an inhibitory segment, usually located in the N-terminus of the peptidase [2,4,5].

In the secretory pathway of eukaryotic cells, a group of serine proteases known as the kexin/subtilisin-like PCs (proprotein convertases) are responsible for the processing of multidomain proteins at specific basic residues into biologically active peptides [6,7]. PCs are themselves synthesized as precursors with 83–106-amino-acid pro-regions that are cleaved in an autocatalytic manner [8] soon after biosynthesis of the enzyme in the ER (endoplasmic reticulum) [9,10].

Studies on one of these enzymes, furin, have contributed greatly to our understanding of cellular and molecular processes, such as enzymic function, protein activation and trafficking, as well as viral and bacterial pathogenesis [11–13]. Furin is a mosaic protein consisting of a series of multifunctional domains. A signal peptide at its N-terminus directs the translocation of the growing peptide chain to the ER and the secretory pathway [14]. The pro-region is involved in folding, activation and inhibition. In furin, this domain is followed by a catalytic region, a P domain which is essential for enzymic activity [15], a cysteine-rich region [16], a transmembrane region and finally a cytoplasmic domain involved in subcellular targeting [12].

By analogy with bacterial subtilisin, the pro-region of furin is believed to serve numerous functions, such as promoting folding, stability, sorting and transiently inhibiting processing activity [17]. A recent study [18] investigated the inhibitory potency of PC

pro-regions, and demonstrated their rather low inhibitory specificities when activities of multiple PCs were compared. While much effort has gone into understanding the mechanism by which pro-regions direct activation of convertases by cleavage at consensus basic sites (Arg-107 and Arg-75 for furin), little is known about the involvement of other conserved residues within the pro-regions of PCs. Previously, the N-terminal pro-region of subtilisin was found to contain three short hydrophobic sequences H1, H2 and H3, all of which are part of larger common motifs that are conserved in numerous unrelated proteins [19–22]. The H1, H2 and H3 motifs are also hot spots for mutations, and it has been speculated that they could play a vital role in folding processes [23].

A recent study by Tangrea et al. [24] identified conserved hydrophobic residues among the pro-regions of PCs. The recent solution structure of the pro-region of PC1 made it possible to classify specific residues into two groups, one forming a hydrophobic core within the pro-region itself and the other forming a hydrophobic patch involved in the binding interface between the pro-region and the catalytic domain of the enzyme, in a similar manner to that seen for the bacterial subtilisins. To gain insight into the function of furin's pro-region, we expressed pro-region mutants and analysed their biosynthesis and enzymic activities. Homology modelling based on recently published data on the structure of the pro-region of PC1 and the catalytic domain of furin along with the results presented herein enable us to provide insight into the mechanisms by which the pro-region acts as a chaperone and inhibitor.

EXPERIMENTAL

Materials

DNA modification and restriction enzymes were obtained from New England Biolabs (Mississauga, Ontario, Canada). All culture

Abbreviations used: ER, endoplasmic reticulum; PC, proprotein convertase; vWf, von Willebrand factor.

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reagents were from Invitrogen (Burlington, Ontario, Canada). The cDNA encoding vWF (von Willebrand factor) was obtained from the Sanquin Blood Supply Foundation, CLB Division (Amsterdam, The Netherlands). The anti-vWF polyclonal antibody was from Dako Diagnostics Canada Inc. (Mississauga, Ontario, Canada).

Cell culture and transfection

QBI-293A cells (293A; derived from the HEK-293 cell line; Quantum Biotechnologies Inc., Montreal, Quebec, Canada) and CHO RPE.40 cells were grown respectively in complete Dulbecco's modified Eagle's medium and RPMI 1640 containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. Subconfluent cells were transfected with 2 µl of Eugene6 reagent (Roche Diagnostics, Laval, Quebec, Canada) per µg of DNA 24 h prior to each experiment.

Recombinant DNA constructs

The 17 pro-region mutants were constructed by site-directed mutagenesis using the Expand High Fidelity kit (Roche Diagnostics). Single mutations in the pro-region of furin were obtained by using the following oligonucleotides (mutated nucleotides are underlined; a silent mutation indicated in bold was used for the screening of mutants): V29A (5'-CAGGGCCAGA-AGGCCTTACCAATACGTGGGCT), T31A (5'-CAGAAGG-TCTTCGCCAATACGTGGGCTGTG), N32A (5'-GAAGGT-CTTACCGCGACGTGGGCTGTG), W34A (5'-CTTACCA-ACACGGCGGCTGTGCGCATC), V36A (5'-CAATACGTGGCTGCGCGCATCCCTGGAG), F54A (5'-CGGAAGCATGGGGCCCTCAACCTGGGCCAAATCTTCGGGGAC), N56A (5'-CATGGGTTCTTCGCCCTGGGCCAAATCTTCGGGGAC), L57A (5'-GGGTTCTTCAACGCGGGCCAAATCTTCGGGGAC), G58A (5'-GTTCTTCAACCTGGGCCAAATCTTCGGGGAC), Q59A (5'-CTCAACCTGGGCGCGATCTTCGGGGAC), I60A (5'-CAACCTGGGCCAAGCCTTCGGGGACTATTAC), D63A (5'-CTCAACCTGGGCCAAATCTTCGGGGCC-TATTACCACTT), Y64A (5'-CTCAACCTGGGCCAAATCTTCGGGGACGCTTACCACTTCTGG), Y65A (5'-CTCAACCTGGGCCAAATCTTCGGGGACTATTGCCCACTTCTGGCATC), H66A (5'-CTCAACCTGGGCCAAATCTTCGGGGACTATTACGCTTCTGGCATCGAG), F67A (5'-CTCAACCTGGGCCAAATCTTCGGGGACTATTACCACGCGCTGGCATCGAGGAG) and H69A (5'-CTCAACCTGGGCCAAATCTTCGGGGACTATTACCACTTCTGGGCTCGAGGAGTGACG). The amplified PCR sequences were subcloned as an *EcoRI*–*ApaI* fragment into an *EcoRI*–*ApaI* site of hfurin/pCI-Neo and were confirmed by nucleotide sequencing.

Metabolic labelling

Metabolic labelling experiments were performed 24 h post-transfection. Cells were washed twice with warm PBS and incubated in Met/Cys-free medium (MEM SelectAmine kit; Life Technologies) supplemented with 10% (v/v) dialysed fetal calf serum, 1 mM L-glutamine and 50 µCi of Expre³⁵S³⁵S Protein Labeling Mix (NEN Life Science Products, Boston, MA, U.S.A.) for 3 h. The culture medium was harvested and adjusted to 1 mM EDTA. The cell layer was washed twice with PBS and cells were lysed with 1 ml of RIPA buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1% Nonidet P40, 0.5% deoxycholic acid and 0.1% SDS) containing protease inhibitors (1 mM EDTA, 1 µM aprotinin, 10 µM pepstatin, 10 µM leupeptin and 0.5 mg/ml

Pefabloc SC). Samples were centrifuged at 15 000 g for 10 min to remove insoluble material. Anti-furin (1:500) or MON150 (1:500; generously provided by W.J.M. Van de Ven, Leuven, Belgium) antibodies were added and incubated overnight at 4 °C [16]. Protein A/G-agarose (Santa Cruz) beads were added and incubated for 1 h at 4 °C. Beads were washed three times with 1 ml of RIPA buffer and labelled proteins were resolved by SDS/PAGE. Gels were treated with ENTENSIFY reagent (NEN Life Science Products), dried and exposed for fluorography. Proteasome inhibitor MG-132 (10 µM) (Roche Diagnostics) was added for the 3 h pulse when required.

Homology modelling and sequence alignment

Sequence alignment of PC pro-regions was performed with ClustalX 1.81. Molecular modelling was performed using the INSIGHTII (2000) suite of programs (Molecular Simulations) on a Silicon Graphics Octane2 workstation. Sequence alignment and co-ordinate transfer was performed with the HOMOLOG module. The potential energy of the resulting model was minimized using DISCOVER with the CVFF force field. The graphic representation was realized using Ribbons 3.14 [CBSE (Center for Biophysical and Engineering), University of Alabama, Birmingham, AL, U.S.A.].

The NMR co-ordinates of the mouse PC1 pro-region [24] were used to create the human furin pro-region model. The catalytic and P domains of human furin were modelled by homology to the crystal structure of mouse furin [25]. All molecular modelling was executed using sequence superimposition. The docking of furin's pro-region on the catalytic domain was performed with reference to the subtilisin *Bacillus* protease N (BNP') structure resolved by Gallagher et al. [26], and was based on charge complementarity and hydrophobic surface topology. A total of 19 surface residues in the catalytic domain were used to create the dimerization interface between the pro- and catalytic domains. The mouse furin and human furin catalytic domains share an identical sequence at the dimerization interface.

RESULTS

Sequence alignment between convertase pro-regions

To verify whether specific residues within the pro-region of furin are conserved among mammalian convertases, we aligned the pro-regions of the seven human mammalian convertases (Figure 1A). Residues predicted to be in the hydrophobic core of convertase pro-regions are boxed, while solvent-accessible hydrophobic residues at the putative binding interface between the pro-region and catalytic domains are highlighted in grey. Figure 1(B) shows the secondary structure conservation between the pro-region of prokaryotic subtilisin BNP' and the pro-region of PC1, based on the crystal structure of BNP' and the solution structure of PC1 [24]. The sequence similarities of the various convertases suggest that the pro-regions have similar overall topologies and structures. To investigate the role of conserved residues in furin pro-region function, three conserved mini-domains were chosen for site-directed mutagenesis to replace 17 selective residues with alanine.

Expression of furin pro-region mutants

To examine the expression of the 17 mutants, QBI-293A cells were transiently transfected with the mutants and were metabolically labelled with [³⁵S]Met/[³⁵S]Cys. Immunoreactive proteins were precipitated in cells and media using an anti-furin antibody

Figure 1 Sequence alignment between convertase pro-regions

(A) Sequence alignment between mammalian convertase pro-regions was performed with ClustalX 1.81. The numbering is for the furin sequence. On the bottom line, highly conserved residues (present in at least five of seven sequences) are in upper-case lettering, while less highly conserved residues (present in at least four of the seven sequences) are in lower case. The 17 mutated amino acids, present in three motifs, are indicated by arrows. The primary and secondary processing sites are underlined. Residues in the hydrophobic core, by analogy with PC1 (according to Tangrea et al. [24]), are boxed, while the solvent-accessible residues of the putative binding interface of PCs are highlighted in grey. (B) Alignment of prokaryotic (subtilisin BNP') and eukaryotic (mouse PC1) pro-region sequences based on secondary structures. Both possess two α -helices and four β -sheets. The primary and secondary processing sites are underlined as in (A).

that recognizes an epitope in the catalytic domain. The three forms detected are shown schematically in Figure 2(A). The zymogen and the highly glycosylated/sialylated processed forms co-migrated at 106 kDa, while the mature form had an apparent molecular mass of 100 kDa. The 81 kDa band represents the shed form of furin, which is secreted into the extracellular space. Figure 2(B) shows that, compared with the characteristic pattern observed for wild-type furin, most of the mutants had the typical 106/100 kDa doublet in cells, together with the 81 kDa shed form in the medium (also detected in cells). We did not detect any immunoreactive bands in cells expressing the I60A-furin and H66A-furin mutants, while those expressing the W34A-furin and F67A-furin mutants produced a single band of 106 kDa.

To determine whether the absence of bands with the I60A-furin and H66A-furin mutants was due to a lack of expression or to protein degradation, biosynthetic labelling experiments were performed using 10 μ M of the proteasome inhibitor MG-132. Figure 3(B) shows that proteasome inhibition resulted in the detection of a 106 kDa band, which would correspond to the zymogen form of furin.

To analyse further the biosynthetic pattern of the W34A-furin and F67A-furin mutants, we used another antibody, MON150, which recognizes an epitope in the pro-region of the enzyme (Figure 3A). This enabled us to distinguish between the zymogen

Figure 2 Biosynthesis of furin pro-region mutants

(A) Schematic representation of the different forms of furin, with the anti-furin antibody recognizing an epitope of the catalytic domain. The different domains of the protein are represented with various patterns. (B) Expression by pulse-chase labelling of the 17 pro-region mutants together with the control (pCl-Neo) and wild-type furin. Cell extract (C) and medium (M) are indicated. The characteristic intracellular furin doublet is indicated by the asterisk, while the arrow indicates the 81 kDa shed form.

Figure 3 Characterization of degraded or activation-defective furin pro-region mutants

(A) Schematic representation of the two epitopes of furin recognized by the MON150 and anti-furin antibodies. (B) Expression of the I60A and H66A mutants (cells (C) and medium (M)) in the absence or presence of 10 μ M MG-132 in a pulse-chase labelling experiment. The zymogen form of furin is indicated by the arrow. (C) Detection of the zymogen form of the W34A and F67A mutants by the anti-furin and MON150 antibodies. The cell extract, medium and zymogen form are indicated as in (B).

and the mature highly glycosylated/sialylated forms that co-migrate at 106 kDa. SDS/PAGE and detection with MON150 revealed the same 106 kDa band as with the anti-furin antibody (Figure 3C), confirming the identity of the form as the furin zymogen with the pro-region still part of the main chain.

Figure 4 Processing ability of furin pro-region mutants toward pro-vWf

(A) Schematic representation of vWf as a substrate for furin. The 325 and 225 kDa forms are shown, along with the different domains of the protein. (B) Processing of vWf precursor by furin pro-region mutants. CHO RPE.40 cells were transfected with pro-vWf vector alone or with furin or pro-region mutants. At 24 h post-transfection, the cells were metabolically labelled for 3 h, after which the medium was harvested. vWf proteins were immunoprecipitated with anti-vWf. The two molecular mass forms are indicated by the arrows.

Processing activity of pro-region mutants

The typical doublet expressed by the majority of pro-region mutants demonstrated the zymogen's capacity for autoactivation. To assess the enzymic activities of the mutants, each was co-transfected into furin-deficient CHO RPE.40 cells along with a cDNA encoding vWf (Figure 4A), a well-documented furin substrate [27,28]. Basal processing of the vWf precursor was observed following pulse labelling of cells transfected with pro-vWf alone. As can be seen in the vWf lane (Figure 4B), the precursor form migrated as a 325 kDa band and was cleaved to the mature 225 kDa form. Every mutant except for I60A-furin and H66A-furin processed the vWf precursor to its mature form. As seen in Figures 2(B) and 3(B), I60A-furin and H66A-furin were degraded early in their biosynthesis. Intriguingly, the W34A-furin and F67A-furin constructs, which only showed the presence of the high-molecular-mass zymogen form (Figures 2B and 3C), retained their proteolytic capacity to process the vWf precursor to its mature form.

Homology modelling of the pro-region of furin

A group of hydrophobic conserved residues in the PC pro-regions is thought to participate in the binding interface between the pro-region and the catalytic domain of the enzyme. To support and complement our biosynthetic data, we used a homology modelling approach. The pro-region of human furin was first modelled on the basis of similarity to the solution structure of the pro-region of mouse PC1. Secondly, the catalytic and P domains of human furin were modelled to the crystal structure of mouse furin [25]. To adequately position the pro-region at the interface of the catalytic domain, we referred to the BNP' subtilisin pro-region/catalytic

Figure 5 Homology modelling of the complex between the pro-region (cyan and orange) and the catalytic domain (red) of furin

The P domain (grey) is also depicted. The structure of furin's pro-region was modelled on to the structure of the PC1 pro-region (residues 28–110; PDB accession no. 1KN6). The catalytic and P domain regions were modelled on to the crystal structure of those of mouse furin (PDB accession no. 1P8J). The interface between the catalytic domain and the pro-region is depicted as a molecular interface (grey). His-66 and Ile-60 side chains are in dark blue. Replacement of these residues by alanine is proposed to weaken the stability of the interface and hamper the chaperone role of the pro-region, leading to an unfolded zymogen and degradation by the proteasome. The autocleavage site (RTKR) and the catalytic domain are also depicted schematically.

complex [26]. This interface is depicted in Figure 5, with the pro-region α -helices in orange. The β -sheet is in cyan, while the catalytic domain is in red, next to the P domain (grey). Residues Ile-60 and His-66 are in dark blue, and two other residues (Leu-57 and Trp-64), which are possibly involved in the binding interface but the mutation of which did not modify the enzyme's biosynthesis, are in cyan. Because of the strategic location of Ile-60 and His-66 at the interface between the pro-region and the catalytic domain, it is possible that their replacement would cause significant perturbations in the conformation of the domain. Such destabilization could prevent the formation of a stable zymogen, leading to an unfolded zymogen state that would be recognized by the proteasome and degraded.

Figures 6(A) and 6(B) represent two angles of furin's pro-region by analogy with the NMR model of the pro-region of PC1 [24]. Residues Trp-34 and Phe-67 are depicted in grey, Phe-54 and Tyr-65 are in yellow, and Val-36 and Asn-56 are in purple. These six residues are present in the hydrophobic core of furin's pro-region by analogy with PC1's pro-region structure, and were mutated in the present study. As seen in Figure 2(B), the W34A and F67A mutations impaired the maturation of furin. Figure 6(A) shows that these residues seem to have a central location in the hydrophobic core, compared with the other four residues, which are in a more peripheral location.

In Figure 6(B), residues Trp-34 and Phe-67, which have bulky aromatic side chains, fill the hydrophobic core of the pro-region. Mutating these residues to alanine would first destabilize the pro-region by diminishing its hydrophobic core, and secondly create cavities (grey area) that would lead to a structural rearrangement and displacement of the Arg-107 cleavage site from the catalytic pocket. Ultimately, the enzyme would remain in an uncleaved zymogen form.

Figure 6 Homology model of furin's pro-region viewed from two angles

(A) Residues Trp-34 and Phe-67 are shown in grey, and are located in the pro-region hydrophobic core. Phe-54 and Tyr-65 are in yellow, while Val-36 and Asn-56 are in purple. These six residues form the hydrophobic core of the pro-region. (B) The Arg-107 cleavage site is depicted at the C-terminus of the pro-region. Replacement of Trp-34 (C) or Phe-67 (D) by Ala will lead to cavity formation. To avoid such cavities, the pro-region would undergo a structural reorganization, i.e. movement of the three strands (black arrows) and the autocleavage RTKR site away from the catalytic site, contributing to the lack of intramolecular autoprocessing of these mutants.

DISCUSSION

Because of the vital role that endopeptidases play in various cellular processes, they must be kept under tight control until they reach their proper compartment. During the biosynthesis of numerous proteolytic enzymes, pro-regions fulfil multiple roles that are of major importance in the production and regulation of the active enzyme. These roles include processes such as folding, activation and inhibition [1,22,29–34]. In the present study, we have identified pro-region residues that are involved in these functions. Many hydrophobic residues in PC pro-regions are conserved among this family of proteases. These residues are also conserved to a certain degree in motifs found in other proteases. Indeed, in structurally and functionally unrelated proteases, two sets of similar motifs, termed N1 and N2 [22], have been shown to contain hydrophobic patches that are important for function. Furin also contains these patches, as illustrated in Figure 1.

Site-directed mutagenesis of selected conserved residues, some within hydrophobic patches, was used to determine their importance in pro-region function. Alanine was chosen as the substitution residue because it eliminates the side chain beyond the β -carbon, but does not alter the main-chain conformation or contribute significantly to electrostatic or steric effects [35]. Overall, of the 17 mutants constructed, two (I60A-furin and H66A-furin) impaired the folding of pro-furin. Based on the solution structure of PC1's pro-region [24], these two residues would be predicted to be involved in the binding interface between the pro-region and the catalytic domain of furin. Since

the I60A and H66A mutations caused rapid degradation of the protein, the pro-region's role as an intramolecular chaperone seems to be affected. Similar residues found in hydrophobic motifs of subtilisin pro-regions are thought to initiate folding through hydrophobic collapse [36]. If the mutated pro-region loses its intramolecular chaperone function, the misfolded protein may be degraded by the proteasome machinery. In fact, in most mammalian cells under optimal nutritional conditions, 80–90 % of protein breakdown occurs via the proteasome pathway and is sensitive to proteasome inhibitors or ATP depletion [37]. It is interesting to note that the residue analogous to Ile-60 in subtilisin's pro-region, Phe-41, is believed to be the central residue that forms the hydrophobic interface with the two surface α -helices of subtilisin [1]. This positioning of furin's pro-region with regard to its catalytic domain is represented in Figure 5. While there is little sequence similarity between bacterial subtilisin and mammalian convertase pro-regions, the residues in the hydrophobic patches of convertases that may be involved in the binding interface share a similar topology with the hydrophobic patch of subtilisin. The conserved residues in this patch are thought to provide a basal level of favourable contacts between pro- and catalytic domains, while other residues may be responsible for specificity [24]. This is consistent with the results of Fugère et al. [18], who showed that PC pro-regions can inhibit the catalytic domain of other mammalian convertases in addition to their own.

The two other mutations of interest in the present study were of residues Trp-34 and Phe-67, which are thought to be involved in the hydrophobic core of the furin pro-region. The replacement

of these putative core residues with alanine would lead to a structural reorganization and destabilization of the pro-region to prevent the formation of cavities, since alanine is much smaller and less hydrophobic than tryptophan or phenylalanine. As shown in Figure 6(B), we suggest that this reorganization is the cause of the inability of these mutants to efficiently undergo zymogen activation. Surprisingly, the W34A and F67A mutants conserved their proteolytic activity, as shown by their ability to cleave the vWf precursor. We cannot absolutely rule out the possibility that an undetected amount of active furin may have been present that could participate in processing pro-vWf. However, if that were the case we would expect that a minimal amount of active furin may not be sufficient to completely process the precursor, resulting in the detection of some precursor form. Yet such a form was not detected. Therefore, since inhibition of furin by its pro-region is probably caused by steric occlusion of the active site, and since these mutations probably reduce the affinity of the pro-region for the catalytic domain, it is conceivable that, in such mutants, the catalytic site is not always obstructed. In fact, Bolognesi et al. [38] reported that it is possible to generate a functional serine protease without limited proteolysis of an activation segment, the pro-region. This is also the case for α -lytic protease and trypsinogen. A model was proposed in which the zymogen form is able to alternate between inactive and active conformations, such that limited proteolysis of the activation fragment forces the zymogen irreversibly into the active state [39]. It should be remembered that the zymogen must possess a certain intrinsic activity just to begin its own activation. Hence our biosynthetic data and modelling support this concept, whereby a zymogen with a pro-region existing in a state of low affinity for its catalytic domain retains intrinsic enzymic activity. Interestingly, recent results obtained on the inhibitory properties of peptides derived from the pro-regions of PC1 and furin have demonstrated how residues His-66 and Phe-67 may well contribute to the affinity of pro-regions for the catalytic domains [40].

In conclusion, we have identified residues in the pro-region of furin that are believed to play a crucial role in the structure of the pro-region itself or in inducing conformational changes in the body of the enzyme. Mutating these residues leads to the proteasome-dependent degradation of furin or to deficiencies in its intramolecular autocatalytic activation. Additional information will be needed to shed light on the relationship between the pro-region and the catalytic domain of furin, and possibly its P domain, in order to adequately define pro-region function.

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