Ectodomain shedding of furin: kinetics and role of the cysteine-rich region

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Abstract Furin, a member of the subtilisin-like pro-protein convertase family, is a type I membrane protein that undergoes ectodomain shedding. Metabolic labeling of cells stably expressing furin demonstrated that the shed form of furin is detected after 30 min. Moreover, sequence analysis revealed that specific residues of the cysteine-rich region of furin aligned with those of tumor necrosis factor receptor, which is also shed. Introduction within furin's cysteine-rich region of mutations that impair TNFR1 shedding also abolished furin shedding. Our results show that shedding of furin occurs rapidly and further suggest that specific cysteine residues may impart a conformation to the enzyme, thereby affecting its susceptibility to proteolysis.

Key words: SPC1; Furin; Ectodomain shedding; Convertase; Cysteine-rich region

1. Introduction

Mammalian subtilisin-like pro-protein convertases (SPCs) are calcium-dependent serine proteases involved in the endoproteolytic cleavage of precursor proteins to yield biologically active polypeptides [1]. The SPC family consists of seven distinct members (SPC1–SPC7) that vary in their tissue and subcellular distribution and in their enzymatic and biochemical properties. Numerous studies on one of these enzymes, SPC1/furin, have greatly contributed to our understanding of cellular and molecular processes such as enzymatic function, protein trafficking as well as viral and bacterial pathogenesis [2,3].

Furin is a mosaic protein consisting of a series of multifunctional domains. At its N-terminus portion, a signal peptide directs the translocation of the growing peptide chain to the endoplasmic reticulum and the secretory pathway [4]. Next, the pro-region initially folds onto the native protein, thereby keeping the enzyme in a zymogen or inactive state. The pro-region is cleaved early in the endoplasmic reticulum by an intramolecular autocatalytic process [5], then associates with the catalytic domain where it guides the protein through the endoplasmic reticulum (ER) and the Golgi apparatus for its eventual enzymatic activation [6]. The catalytic region that follows possesses the characteristic catalytic triad (Asp, His, Ser) found in the subtilisin family of serine proteases. C-terminal to the catalytic domain is the P domain that has been found to be essential for the enzymatic activity [7]. The cysteine-rich region (CRR) has no known role and is followed by a trans-membrane region that anchors the enzyme in the membrane of the TGN or the cell surface. Finally, the cytosolic tail contains the information necessary for furin's sorting to various intracellular compartments [3,4,8].

It has been demonstrated that furin can process an extensive range of precursor proteins in the TGN/biosynthetic pathway but also in the endocytic pathway, at the cell surface and possibly within the extracellular matrix following shedding. Apart from numerous studies describing the processing of bacterial precursor molecules by cell surface or shed SPC1, proteins such as profibrillin, a large extracellular matrix glycoprotein, have been shown to be potential extracellular substrates for furin [9], although it remains to be determined whether proteolytic cleavage is performed by the membrane anchored or the shed form of the enzyme. Recently, it has been proposed that the TGF-β1 and TGF-β2 precursors, known to be processed by furin in the TGN [10,11], may also be cleaved extracellularly by a secreted or shed form of the enzyme [12].

Ectodomain shedding is a cellular process whereby the extracellular domains of trans-membrane proteins are proteolytically cleaved, often at the cell surface [13]. This post-translational modification is known to modulate biological function by releasing such proteins as growth factors, enzymes and receptors. For example, tumor necrosis factor receptor type 1 (TNFR1) will normally undergo shedding in order to release a soluble moiety able to bind TNFα. This will reduce the cell's sensitivity to TNFα [14], thus regulating the inflammatory process. Although some shedding proteases, such as tumor necrosis α-converting enzyme (TACE/ADAM17) [15], have been characterized, there are evidences stemming from the use of TACE knockout mice that suggest the existence of non-TACE shedding mechanisms [16].

Although many studies have reported shedding of furin, few have thoroughly characterized this process. Here we demonstrate that furin shedding occurs intracellularly and may

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Abbreviations: ACE, angiotensin-converting enzyme; Amc, 7-amido-4-methylcoumarin; CRR, cysteine-rich region; ER, endoplasmic reticulum; SPC, subtilisin pro-protein convertase; TACE, proTNF-α-converting enzyme; TGN, trans-Golgi network; TMD, trans-membrane domain; TNFR, tumor necrosis factor receptor

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depend on the integrity of the conformation of the enzyme's CRR. These results add to our understanding of the molecular determinants involved in targeting furin to various cellular locations.

2. Materials and methods

2.1. Cell culture, transfection and clonal cell line isolation

QBI-293A cells (293A, Quantum Biotechnologies, Montreal, QC, Canada), derived from the HEK293 cell line, were grown in complete DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Sub-confluent 293A cells were transfected with 2 μl of Fugene6 reagent (Roche Diagnostics, Laval, QC, Canada) per μg of DNA 24 h prior to each experiment. FLAG-tagged furin expressing stable cell line was obtained from a population of 293A cells that was selected for 3 weeks with G418 (0.8 mg/ml, Life Technologies, Burlington, ON, Canada) using the cloning ring method. Cells were screened by immunoblot with anti-furin and monoclonal M1 antibodies. The cell line termed C4-293A corresponds to a stable non-clonal population of cells.

2.2. Recombinant DNA constructs

FLAG-tagged furin was constructed by site-directed mutagenesis using the Transformer mutagenesis kit (Invitrogen, San Diego, CA, USA) and an oligonucleotide (5′-GCTCCTGGTACACGTCCTTGTCATCGTCGTCCTTGTAGTCCCGTTTAGTCCGTCGC) to insert the FLAG epitope (AspTysLysAspAspAspAspAla) after Arg^{107} between the pro-region and the catalytic domain. The cDNA encoding furin (without the FLAG epitope) was sub-cloned as an *Eco*RI–*Eco*RV fragment into *Eco*RI–*Sma*I of pCI–Neo. An *Apa*I–*Not*I fragment was used to replace a similar insert in pCI–Neo/hSPC1 to yield the corresponding FLAG constructs. Single mutations in the CRR of furin were obtained by site-directed mutagenesis as above using the following oligonucleotides: C610R (5′-CCAGAAGAGCCGTGTCCAGCAC, mutated codon is underlined), C614Y (5′-GTCCAGCACTACCCTCCAGGC), S639M (5′-CATCCGGGCCATGGTGTGCGCC), C641F (5′-GCCAGCGTGTTCGCCCCCTG), and C675R (5′-GGAGCAGACGCGTTCCCGGC).

2.3. Metabolic labeling and shedding quantification (shedding assay)

Metabolic labeling experiments were performed 24 h post-transfection or when cells reach 90% confluence. Cells were washed twice with warm PBS and incubated in Met/Cys-free medium (MEM Select-Amine kit, Life Technologies) supplemented with 10% dialyzed FCS, 1 mM L-glutamine, and 50–100 μCi of Expre^{35}S^{35}S (NEN Life Science Products, Boston, MA, USA) for the indicated period of time. Chase was done in complete medium. Cell layer was washed twice with PBS and cells were lyzed with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM $CaCl_2$, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS) containing protease inhibitors (1 μM aprotinin, 10 μM pepstatin, 10 μM leupeptin and 0.5 mg/ml pefabloc SC). Samples were centrifuged at $15\,000\times g$ for 10 min to remove insoluble material. M1 (10 μg/ml, Sigma) antibody was added and incubated overnight at 4°C. Protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) beads were added and incubated for 1 h at 4°C. Beads were washed three times with 1 ml of RIPA buffer and labeled proteins were resolved by SDS–PAGE. Gels were treated with ENTENSIFY reagent (NEN Life Science), dried and exposed for fluorography. For shedding quantification, gel bands were excised and mixed with liquid scintillation cocktail to determine the amount of radioactivity of each band. A gel slice of similar dimension excised from control sample was used as background value and subtracted to yield specific radioactivity of each sample. BFA (5 uM) and monensin (5 uM) were added to the labeling mixture where indicated.

2.4. Furin enzymatic assays

Proteins secreted from transfected cells were accumulated in Dulbecco modified Eagle medium (DMEM)-based serum-free medium and 0.1 ml was used in a fluorogenic assay using 100 μM t-BocRVRR-Amc as substrate in assay buffer [200 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 1 mM $CaCl_2$, 1 mM β-mercaptoethanol] at 37°C. Hydrolyzed 7-amido-4-methylcoumarin (Amc) was measured at $EX_\lambda = 380$ nm and $EX_\lambda = 460$ nm.

2.5. Homology modeling

All the molecular modeling was performed using the INSIGHTII (2000) suite of programs (Molecular Simulations) on a Silicon Graphics Octane2 workstation. Sequence alignment and coordinate transfer was performed with the HOMOLOGY module. Ψ angle torsion and mutations C614Y/C641F were performed using the BIOPOLYMER module and the potential energy of resulting model was minimized using DISCOVER with the CVFF force field.

3. Results

3.1. Characterization of the shedding process

To examine the kinetics of furin shedding, we metabolically labeled proteins of C4-293A cells stably expressing a FLAG-tagged furin followed by immunoprecipitations using the M1

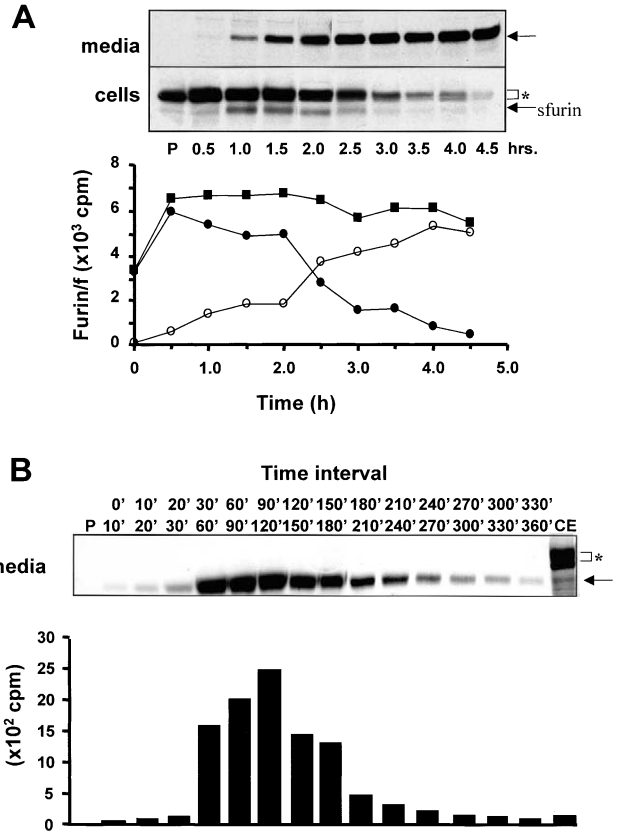

Fig. 1. Kinetics of furin ectodomain shedding. A: Accumulation of shed furin in culture medium and depletion of cellular furin. 35-mm plates of C4-293A cells were pulse-labeled with [^{35}S]Met/Cys for a period of 30 min and chased in complete medium for the indicated period of time (0–4.5 h). Labeled proteins from cell extracts prepared in RIPA buffer or cultured medium were immunoprecipitated with M1 antibody. The amount of radioactivity immunoprecipitated was quantified by liquid scintillation counting and plotted as a function of chase time. Radioactivity corresponding to the shed furin (○), cellular furin (●) and total (■) from cells and media are presented in cpm. The initial increase in cellular furin is due to processing of the zymogen that is not detected by the M1 antibody. Analysis by measurement of the total amount of immunoprecipitated furin (in cpm) revealed that only 5% of total radioactivity was lost during the 4.5-h chase period. B: Time course of furin shedding. Clonal cells expressing FLAG-tagged furin were pulse-labeled with 500 μCi of [^{35}S]Met/Cys in a 90-mm petri dish and chased in radioactive-free medium. Medium was harvested initially at every 10 min, then every 30 min and replaced with fresh medium every time over a 6 h period. Final cell extract was prepared in RIPA buffer, samples were immunoprecipitated with M1 antibody and quantified as in A. * indicates the mature membrane-bound form of furin.

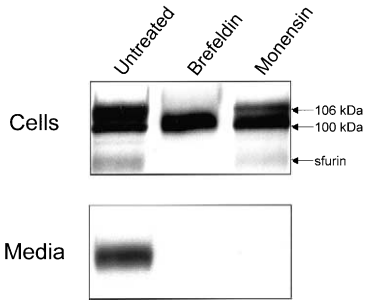

Fig. 2. Furin is rapidly shed in the secretory pathway. Cells were treated as in Fig. 1B in the presence of 5 μM BFA or 5 μM monensin, except that cells were pulsed for only 3 h. sfurin, shed furin.

antibody, which only detects the mature form of the enzyme. Figure 1Ashows the characteristic furin doublet (100 and 106 kDa) in cells representing processed enzyme along with an 81-kDa form. This entity is the shed form of the enzyme also detected in the media. The kinetics of shedding was then examined. Following the pulse period (30 min), we either accumulated secreted proteins over a 0.5 to 4.5-h chase period (35-mm plates, Fig. 1A) or replaced the medium initially at 10-min intervals, then at 30 min intervals (90-mm plates, Fig. 1B). The 81-kDa shed form was detected intracellularly after the 30-min pulse (Fig. 1A, lane P), peaking between 1–1.5 h and accumulating intact in the media over the next 3 h. Indeed, during the 4.5-h period no other forms of furin were detected with the M1 antibody, suggesting that it was not processed into smaller forms. Over this time period, we determined that shedding is the major pathway by which membrane-bound furin is post-translationally modified since less than 5% of total furin immunoreactivity is lost through other processes (see plot Fig. 1A). Importantly, time-course studies showed that we detected soluble furin in the media after only a 10-min chase (Fig. 1B) with a maximal secretion rate observed between 1h and 2h.

To establish where furin shedding takes place, we treated the C4-293A cell line with the Golgi-disturbing agents Brefeldin A (BFA) and monensin (Fig. 2). Pulse-labeling analysis revealed that BFA, which inhibits protein transport between the ER and the Golgi apparatus, does not impede production of the mature form of the enzyme (detection with M1) but affects the glycosylated/sialylated state of the enzyme as demonstrated by the absence of the 106-kDa form in cells. Moreover, production of shed furin was completely abolished (cell and media), whereas treatment with monensin, which inhibits post-Golgi transport, only affected secretion of the shed form and not its production. Taken together, these results clearly identify the Golgi network as the major site of furin shedding.

3.2. *Furin cysteine residues of the CRR align with those of TNFR1*

Using the BLAST search engine, we searched the GenBank for proteins having homologous regions to the CRR of furin (residues 596–675). Fig. 3 depicts the alignment of the CRR of furin with the CRR of tumor necrosis factor receptor (TNFR) family members TNFR1 and TNFR2. We also found patterns of sequence similarity with the CRRs of insulin-like growth factor receptor and the erbB receptor protein-tyrosine kinase (not shown). The crystallographic structure of the extracellular domain of TNFR1 [17] has enabled the assignment of those cysteine residues participating in disulfide bridges. Although the overall similarity of sequence is low within this region, it can be seen that of the six pairs of cysteine residues forming disulfide bridges in the 89-residue CRR of furin, four pairs are 'conserved' within TNFR1 and TNFR2. Based on the alignment of the CRR of furin with TNFR1, Cys^{641} (which could correspond to C80 of TNFR1) may establish a disulfide bond with Cys^{614}. Following reports of mutations within this region [18] that affect shedding, we introduced those mutations at those conserved positions in the CRR of furin to ascertain the role of those residues in the shedding process.

3.3. *Impaired shedding of furin with the C614F and C641F mutations*

Five furin mutants corresponding to the TNFR1 naturally occurring mutants [18] were constructed (C610R, C614Y, S639M, C641F, C675R). These proteins and wild-type,

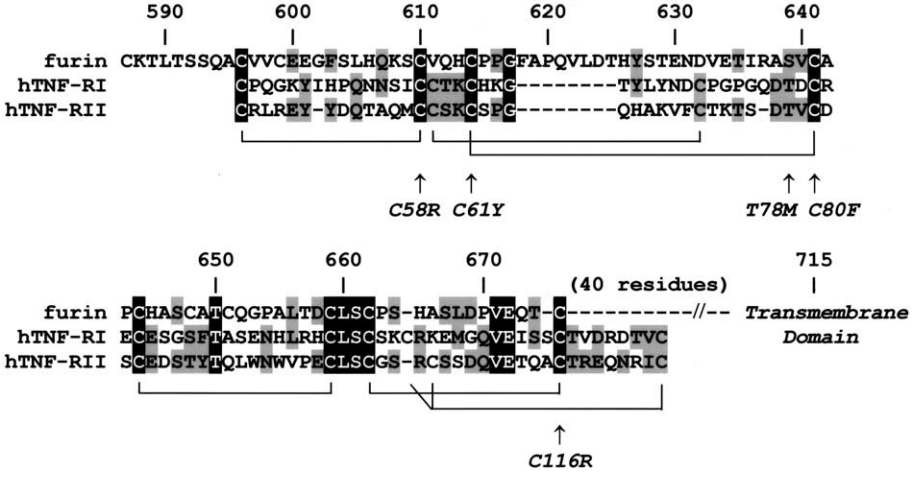

Fig. 3. Sequence alignment of the CRR of human furin with TNF receptors. Sequences from furin (residues 587–675), TNF-RI (residues 44–125) and TNR-RII (residues 18–97) were aligned using hierarchical clustering [35]. Identical amino acids are shadowed and semi-conserved or amino acids of similar properties are grayed. Disulfide bridges found in TNF-Rs, as determined by the crystallographic structures, are drawn below the alignment. Arrows indicate the five point mutations in TNF-RI related to the autosomal periodic fever syndrome identified by McDermott and colleagues [18].

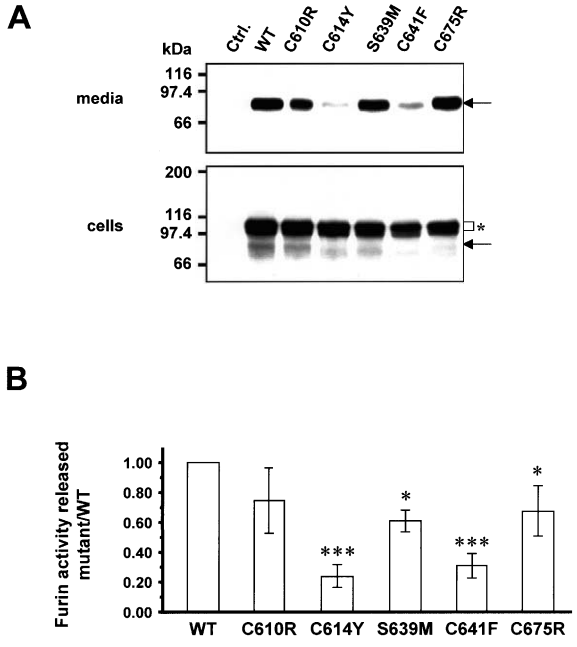

Fig. 4. Analysis of point mutations in the CRR of furin and the effect on shedding. A: Mutations in the CRR of furin impair shedding. 293A cells were transiently transfected with an empty plasmid (Ctrl), wild-type FLAG-tagged furin (WT) or the various mutant cDNAs. Cells were pulse-labeled with [^{35}S]Met/Cys for 3 h, media was harvested and cell extracts were prepared in RIPA buffer followed by immunoprecipitation with M1 antibody. The arrows indicate the shed form whereas the asterisk indicates the mature membrane-bound forms of furin. B: Measurement of enzymatic activity of shed furin. 293A cells were transfected as in A with the various cDNAs. Proteins were accumulated in serum-free medium for 30 h and assayed as described in Materials and Methods. Each assay was normalized with a pulse-labeling experiment following media removal. Media was harvested and cell extracts were prepared in RIPA buffer, immunoprecipitated with M1 antibody, and radioactive bands were counted for radioactivity content by liquid scintillation counting. For each assay, background enzymatic activity from control cells was subtracted from all obtained values and normalized with furin expression level as determined by the labeling experiment of each sample. Data are compared to wild-type samples.

flagged furin were transiently expressed in 293A cells, which were then metabolically labeled with [^{35}S]Met/Cys for 3 h and harvested immediately. Fig. 4A shows that immunoprecipitation of cell lysates with the M1 antibody led to the detection of the characteristic doublet at 106 kDa and 100 kDa, corresponding to the two glycosylated/sialylated states of the mature form of the enzyme. This indicated that mutations within the CRR of furin did not affect its capacity for autocatalytic activation. Immunoprecipitation of the culture media enabled us to detect the shed form of the enzyme. This form appeared in the media of cells transfected with the C610R, S639M and C675R mutants at levels that were similar to those found in the media of cells expressing the wild-type enzyme. However, only a weak signal was detected in the media of cells transfected with the C614Y and C641F constructs. Based on the alignment with the TNF receptors, these two cysteine residues could potentially form a disulfide bridge. The diminished signal revealed by immunoprecipitation was confirmed by measuring the enzymatic activity in the media of transfected cells (Fig. 4B). Indeed, media originating from the C614Y and C641F mutant expressing cells exhibited a marked reduction in their capacity to cleave a furin fluorogenic substrate. Together, these results suggest that specific cysteine residues within the CRR are possibly involved in imparting a conformation to this region of the molecule, enabling the eventual recognition and cleavage by the sheddase.

4. Discussion

We and others had previously demonstrated the capacity of furin to undergo proteolytic shedding resulting in a soluble, secreted protein of 81 kDa [19–22]. Here, we demonstrate that this process may occur in the Golgi network. Detection of the 81-kDa form intracellularly occurs after 30 min and is also found early in the media. Considering that cleavage of the pro-region of furin has been found to occur within a $t1/2$ of 10 min in the ER and is necessary for export [23], this would imply that the shedding process may take place in the Golgi apparatus. This is confirmed with our results using brefeldin A. Moreover, this is also supported by treatment with monensin, a known inhibitor of post-Golgi transport, which enables shedding but not secretion of the soluble form. However, we cannot exclude the possibility that (1) the use of BFA could indirectly affect the activation of the sheddase in the Golgi, thereby leading to lack of shedding or (2) that cell-surface furin may also be shed perhaps by family of proteases other than those found in the Golgi. Because dissociation of the pro-region from membrane-bound furin has a $t1/2$ of 105 min, it is conceivable that the shed form reaches the late secretory pathway at a faster rate, thereby resulting in its efficient secretion. Interestingly, the present findings resemble those recently obtained for another mammalian subtilase, SKI-1, whereby it was shown that shedding would occur following exit from the ER [36].

To date, five distinct metalloproteases have been shown to be involved in shedding membrane proteins (TACE/ADAM17, kuzbanian/ADAM10, ADAM9, ADAM19 and MMP-7) and most, if not all, perform the shedding process at the cell surface. Mutations at or near the cleavage site of various proteins have led to the conclusion that the critical parameter for shedding of specific proteins by a given metalloproteases is a relaxed sequence specificity of the enzyme along with the conformation and/or length of the stalk region rather than conservation of the amino acid sequence [24]. However, a recent report describing shedding of the angiotensin-converting enzyme (ACE), has demonstrated that another interpretation of the shedding process could be that an enzyme of the serine protease family could be implicated as a sheddase and that this event would occur in the ER [25]. Other reports have shown how this stalk region is sensitive to mutations yielding increased levels of shed ACE [26]. Although the enzyme responsible for furin shedding has not yet been identified the site at which cleavage occurs is Arg^{683} ([27] and unpublished data), reminiscent of an ACE or ACE-like sheddase. Indeed, it has been shown that the site of cleavage of ACE by its secretase is Arg^{1203}, 27 residues on the extracellular side of its trans-membrane domain, similar to Arg^{683} of furin, which is shed 32 amino acids from the membrane. Clearly, additional experiments will be needed to identify the SPC1 sheddase.

We hypothesized that the CRR of SPC1 could play a role in shedding following reports that germline mutations in the extracellular domain of TNFR1 (more specifically within its CRR) defined a family of dominantly inherited autoinflamma-

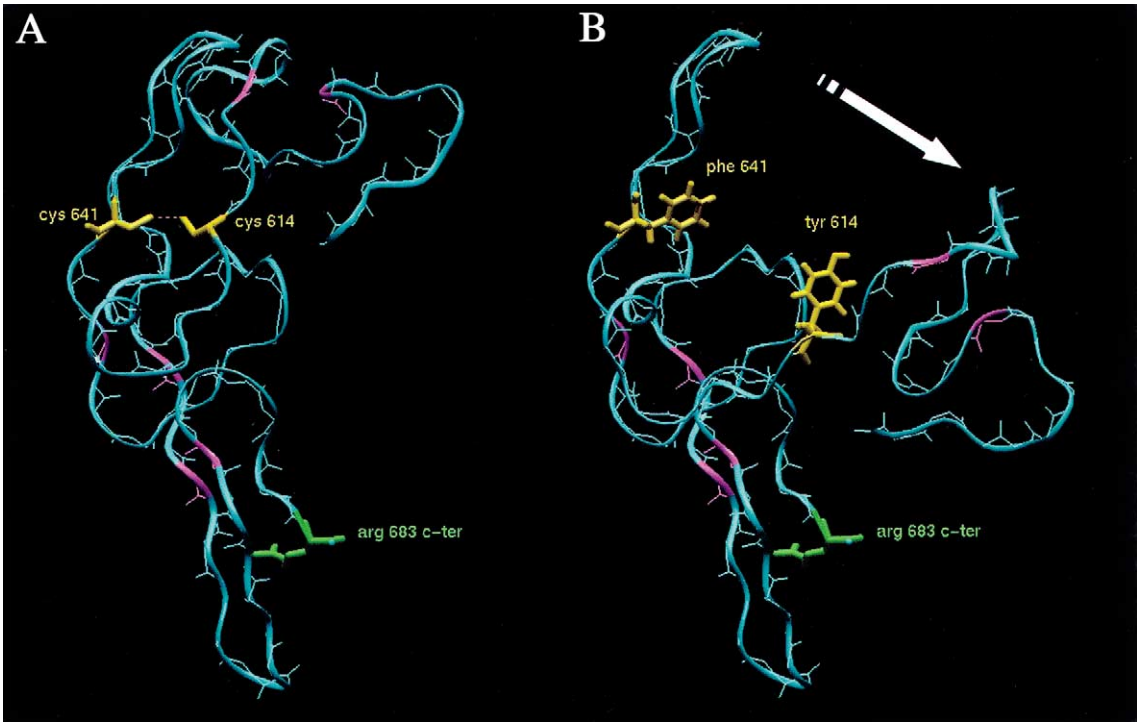

Fig. 5. Homology modeling of furin's CRR. A: Intact CRR of furin. B: CRR of C614Y/C641F mutation. The abolished putative disulfide bridge between C614 and C641 could lead to important conformational changes such as displacement (arrow) of the N-terminal domain. This in turn may impede the sheddase's ability to interact with an intact CRR thereby enabling cleavage at R683. Furin model A was obtained by alignment of gapped sequences of the furin's CRR (residues 587–683), onto the hTNF-RI CRR (residues 44–133, PDB access No. 1EXT). Reorientation of N-terminal domain of B model was obtained by torsion of Ψ angle of proline 620 from 147° to −150°. Cysteine residues participating in disulfide bridges are represented in purple.

tory disorders [18]. More specifically, of the six different mutations of TNFR1 found in the affected families, five disrupted conserved extracellular disulfide bonds (illustrated in Fig. 3). It had been proposed that such substitutions would significantly alter the structure and, hence, the biosynthesis/shedding of TNFR1. One particular mutation, C80F, led to a significant increase in the levels of membrane TNFR1 and reduced clearance, suggesting the role of this region of the receptor in conferring a proper conformation and enabling the eventual cleavage of the ectodomain.

Our results suggest that furin carries structural information within its CRR that facilitates shedding of its ectodomain. Interestingly, another shed convertase, PC6B, also contains an extensive CRR while PC7, which is a type I membrane convertase, is not shed and does not contain a CRR [28,29]. The CRR could participate in the shedding process by imparting a conformation to furin facilitating recognition and cleavage by its sheddase. Mutating those cysteine residues (Cys^{614} and Cys^{641}) that potentially form a disulfide bridge could lead to a disruption of the integrity of the CRR, leading to displacement of the main chain toward Arg^{683} (Fig. 5). The resulting steric hindrance would reduce the accessibility to the sheddase and drastically affect cleavage at this site.

CRRs exist in a variety of proteins, many of which are found at the cell surface. Although the role of these domains has remained rather elusive, some findings have revealed how CRRs could be considered either as functional regions involved in cell adhesion [30], in interacting with specific target proteins [31,32] or in influencing conformation enabling dimerization or shedding of membrane proteins [33].

There have been very few reports describing secreted forms of endogenous furin in cultured cells. In epidermis, furin was detected as two molecular weight forms, one without the trans-membrane domain, suggesting occurrence of post-translational cleavage to produce a soluble enzyme [34]. Moreover, glioma cells that produce TGF-β have been shown to release furin into media as a 98-kDa band [12]. These findings suggest that an extracellular form of furin could be directly involved in processing precursor proteins such as pro-TGF-β, pro-fibrillin and pro-fillagrin. It is still unclear, whether the proteolytic processing of these proteins is the result of the membrane-anchored form of furin or its shed entity. Use of 'unsheddable' mutants of SPC1, such as those described here, could aid in delineating the role of the cleaved, soluble form of this convertase.

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