INTRODUCTION

Furin, also called PACE (paired basic amino acid-cleavage enzyme), was the first endoprotease identified among the family of mammalian subtilisin-like proprotein convertases (SPCs or PCs), which have been implicated in the endoproteolytic maturation of inactive protein precursors at characteristic motifs, containing basic amino acid residues within the secretory pathways [1,2]. Presently, seven distinct members comprise this family, including furin, PC1 (also known as PC3), PC2, PACE4, PC4, PC5 (also termed PC6) and PC7 (also called LPC or PC8). Each of the endoproteases exhibits a unique tissue distribution. Functional redundancy of various PCs in a given tissue or cell type may occur [3,4].

Furin, a glycoprotein, is expressed ubiquitously in all mammalian tissues and cell lines examined and is capable of processing the precursors of a wide range of bioactive proteins, such as growth factors, hormones, plasma proteins, receptors, viral envelope glycoproteins and even bacterial toxins within the constitutive secretory pathway as well as upon endocytosis [5]. It is a calcium-dependent serine endoprotease structurally composed of several moieties: the signal peptide, the propeptide, the catalytic domain, the middle (also termed homo-B or P-) domain, followed by the cysteine-rich and the transmembrane domains as well as the cytoplasmic tail. The transmembrane domain anchors furin in the membranes of the trans-Golgi network. In addition, its trafficking between trans-Golgi network, endosomal compartments and the cell surface has been described [6,7]. In the cytoplasmic tail, a number of amino acid motifs have been shown to be involved in targeting furin to the specific compartments as well as retrieving it from the cell surface [8–12]. Trafficking regulation requires the interaction of furin with additional proteins, e.g. actin-binding protein 280 (ABP-280) and phosphofurin acidic-cluster-sorting protein 1 (PACS-1) [13,14], as well as phosphorylation events of cytosolic tail motifs [10,15–17].

Interestingly, furin has been reported to form a naturally truncated and, hence, secreted form called ‘shed’ furin, which exhibits functional activity even though it lacks the transmembrane domain and the cytoplasmic tail [18–22]. Findings reported previously, based on experimental C-terminal truncations of full-length furin, implied that the ‘shed’ cleavage site may be located in the vicinity of and N-terminal to Leu298 [18]. The identification of the amino acid residues crucial to shedding and the determination of the C-terminus of shed furin, both of which are described in this report, improve our understanding of the shedding process and will aid the elucidation of its underlying physiological role.

MATERIALS AND METHODS

Furin mutant expression plasmids

Substitution and deletion mutants were introduced into mammalian expression vector pCMV-furin wt (previously also termed pCMV-rFurin [794aa]) (short 3’ UTR) [18] using standard PCR cloning procedures [23]. This plasmid contains the entire furin cDNA, encompassing the 2382 bp furin-coding region as well as approx. 90 bp of the 5’ non-translated and 400 bp of the 3’ non-translated region, and mediates furin expression by its cytomegalovirus (CMV) promoter/enhancer. The resulting furin variants are displayed in Figure 1. Mutant constructs were denoted by first naming the native amino acid, using single-letter code, followed by its position within the furin coding sequence, and ending with the amino acid introduced instead.

Plasmid pfur/3xG-FIIa-2xG-6xH-3xG, used to determine the exact C-terminus of recombinant shed furin (shed furin), was constructed employing two subsequent inverse PCR steps. Between Ser295 and Gly296 of full-length furin, a thrombin-cleavage site was inserted, followed by a hexahistidine stretch. The latter served to facilitate affinity purification via a Ni2+-nitrilotriacetate (Ni-NTA)-agarose. These moieties were separated by glycine residues to provide for steric flexibility. Employing template pBS/fur1176, containing the internal 1176 bp BamHI fragment of the wild-type furin cDNA in the pBS SKI (+) vector (Stratagene), in combination with the sense primer 5379 (5’-AGTCAGGCCCTGTTGTTGCGAGG-3’) and the reverse primer 5337 (5’-GGACGTGAGGGTCTTGC-

Abbreviations used: PC, proprotein convertase; CMV, cytomegalovirus; Ni-NTA, Ni2+-nitrilotriacetate.

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For transfection, HEK-293 fibroblast cells (ATCC CRL 1573). The cells were routinely grown in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) medium (Life Technologies) with 10% fetal calf serum (full medium). Permanent cell clones were selected by the addition (1:1) medium (Life Technologies) with 10% fetal calf serum (full medium). Permanent cell clones were selected by the addition of 10 μg/ml hygromycin B (Roche) to the cell culture medium containing 30 μmol of each primer, 200 μM of each dNTP, 2 mM MgSO₄ in the supplied 10 × PCR buffer and 2.5 units of Vent® DNA polymerase (New England Biolabs) at 94 °C for 30 s, at 55 °C for 30 s and at 72 °C for 45 s up to 4 min (according to the expected length of the amplification product). The final extension at 72 °C was prolonged to 5 min. PCR fragments were purified using the QIAEX II Gel Extraction Kit (Qiagen) according to the supplier's instructions.

**Transfection, cell culture and protein harvest**

Furin constructs were transiently or stably expressed in HEK-293 fibroblasts (ATCC CRL 1573). The cells were routinely grown in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) medium (Life Technologies) with 10% fetal calf serum (full medium). Permanent cell clones were selected by the addition of 100 μg/ml hygromycin B (Roche) to the cell culture medium 48 h after transfection.

For transfection, cells were grown to 50–75% confluency on 5 cm culture dishes (Costar) and transfected by calcium phosphate co-precipitation as described previously [24]. Transient transfections were carried out with 20 μg of expression plasmid. For the establishment of stable HEK-293/fur-IIa/His cells, 20 μg of expression plasmid pfur/3xG-FIIa-2xG-6xH-3xG was co-precipitated with 1 μg of selection plasmid pCMV-hyg, mediating resistance to hygromycin B. Resistant clones were isolated 2 weeks after transfection and stabilized by subcloning under selective pressure.

Recombinant protein was harvested once the cells had reached confluency. The confluent cells were washed twice with PBS then incubated with serum-free full medium. Medium was changed every 24 h. Collected medium was clarified by centrifugation. For cell-number determination, the adherent cells were trypsinized, washed with PBS and counted in a CASY counter (Schärfe Systems) employing a 30 μm capillary. Cell extracts were prepared by lysing the cells at a concentration of 5 × 10⁶ cells/ml of lysis buffer, containing 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.5% Triton X-100. After incubation for 30 min at 4 °C, lysates were clarified by centrifugation for 15 min at 10000 g.

**Western Blotting**

The samples were reduced and denatured, and the proteins resolved by SDS/PAGE on 4% stacking/8 or 10% separation gels and visualized by Western blotting as described in [25]. For the detection of furin molecules, murine monoclonal antibody MON-148 (Alexis) directed against the catalytic domain of furin was used. After concentrating the conditioned media 10 × by speed-vac centrifugation, proteins containing histidine tags were visualized employing a murine monoclonal anti-Penta-His antibody (Qiagen). Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) served as the second antibody.

**Analysis of furin activity**

Functional activity of shed furin molecules was determined using a fluorogenic substrate as described previously [25].

**Purification of His-tagged furin**

Conditioned medium derived from HEK-293/fur-IIa/His cells was repeatedly harvested every 24 h for several days and furin...
activity was determined. Supernatants were pooled and incubated with purified 2.5 units of thrombin/ml (Sigma) for 1 h at 37 °C. Completion of cleavage was verified by Western blotting and visualization of the His-tagged cleavage product with anti-His antibody and the disappearance of the uncleaved shed furin form. Subsequently, the reaction mixture was dialysed (SnakeSkin™ dialysis tubings, molecular-mass cut-off \( \approx 3.5 \text{ kDa} \); Pierce) against buffer L (300 mM NaCl/50 mM NaH₂PO₄, pH 8.0/10 mM imidazole), and 6 × His-tagged protein was bound to Ni-NTA-agarose (Qiagen) by incubating 20 ml of conditioned medium and 0.5 ml of resin overnight at 4 °C on an end-over-end shaker. Bound material was washed three times with buffer L containing 20 mM imidazole, and incrementally eluted with 1 ml of buffer L containing 50, 100, 200 and 500 mM imidazole. Eluted material was dialysed against PBS and concentrated by speed-vac centrifugation, and its purity and identity determined by SDS/PAGE followed by silver staining and Western blotting.

**Protein sequencing and MS analysis**

Concentrated 6 × His-tagged protein mixture was separated by reversed-phase HPLC using a Jupiter 5μ column (Phenomenex; C-18, 2 × 150 mm) fitted to a Hewlett Packard 1100 HPLC essentially as described in [26]. N-terminal amino acid sequence analysis was performed using standard Edman chemistry with an Applied Biosystems model 477A Protein Sequencer as described in [27]. MS analysis was performed on a Micromass Platform II mass analyser equipped with a Nanoflow electrospray ionization source according to Medzihradszky et al. [26].

**RESULTS**

**Amino acid residues between Arg⁶⁷⁷ and Gln⁶⁸⁸ of full-length furin are crucial to shedding**

Recent findings suggest that the intramolecular cleavage site leading to shed furin is located in the vicinity of and N-terminal to Leu⁷⁰⁸. Expression of human furin experimentally C-terminally truncated after Leu⁷⁰⁸ resulted in the production of two furin protein bands detectable by Western blotting of the conditioned medium of transfected cells [18]. The larger molecule is the truncated furin lacking the transmembrane domain, whereas the slightly smaller molecule is equal in size to shed furin.

In order to more precisely map the region where the ‘shedding cleavage’ occurs, full-length furin mutant molecules were designed which lacked internal stretches of amino acids N-terminal to Leu⁷⁰⁸. Clusters of 10, 20, 30 and 40 consecutive amino acids located roughly between the cysteine-rich region and the transmembrane domain were eliminated, encompassing amino acids from positions 698–707 (plasmid pΔ698–707), 688–707 (pΔ688–707), 678–707 (pΔ678–707) and 668–707 (pΔ668–707; see Figure 1). These mutant furin constructs (whose expression was mediated by the CMV promoter/enhancer system) were analysed for their ability to form shed molecules by transient transfection into HEK-293 cells. In the conditioned media of the cells transfected with those plasmids encoding furin mutants Q678A/S679A/D688–707, Q678A/S681A/D688–707, Q678A/S685A/D688–707 and P686A/P687A/D688–707, shed furin could readily be detected (Figure 3A, lanes 7, 6, 3 and 2), respectively. In contrast, variant S682A/R683A/D688–707 exhibited severely reduced shedding (Figure 3A, lane 5). Similarly, furin variant R683A/D688–707 produced only trace amounts of shed furin (Figure 3A, lane 4). Equal amounts of intracellular furin were found for all constructs, independent of the differences in generation of shed furin (Figure 3B). These data strongly suggest that Arg⁶⁸³ plays the prime role in shedding.

**Arg⁶⁸³ represents the prime determinant for shedding**

In order to more precisely identify those amino acids within the Gln⁶⁸⁸ to Pro⁶⁸⁷ region that are pivotal to shedding, successive pairs of amino acids were systematically replaced by alanine residues (see Figure 1). As the parent construct used for establishing this series of molecules, pΔ668–707 was chosen. pΔ668–707 contains the largest internal deletion still capable of generating wild-type amounts of shed furin (Figure 2A, lane 4). The impact of the substitutions on furin shedding was evaluated by transient transfection into HEK-293 cells. In the conditioned media of the cells transfected with those plasmids encoding furin mutants Q678A/S679A/D688–707, Q678A/S681A/D688–707, Q678A/S685A/D688–707, E684A/S685A/D688–707 and P686A/P687A/D688–707, shed furin could readily be detected (Figure 3A, lanes 7, 6, 3 and 2), respectively. In contrast, variant S682A/R683A/D688–707 exhibited severely reduced shedding (Figure 3A, lane 5). Similarly, furin variant R683A/D688–707 produced only trace amounts of shed furin (Figure 3A, lane 4). Equal amounts of intracellular furin were found for all constructs, independent of the differences in generation of shed furin (Figure 3B). These data strongly suggest that Arg⁶⁸³ plays the prime role in shedding.

**Ser⁶⁸² is the C-terminus of shed furin**

Since endoproteolysis commonly occurs C-terminal to arginine residues, Arg⁶⁸³ may represent the C-terminal end of shed furin. Alternately, Arg⁶⁸³ could serve only as a determinant for the specificity and/or efficacy of the shedding process. In order to determine its actual C-terminus, shed furin was subjected to MS.
to provide conformational flexibility and accessibility of the
Both of these moieties were flanked by glycine residues in order
contain the motif LVPRGS, a cleavage site for the endoprotease
positions 387, 440 and 553 [28,29]. This linker was designed to
influence shedding) and the postulated glycosylation sites at
(but sufficiently remote from Arg
's)

Figure 3 Fine mapping of the 'shedding site' in furin by individual amino acid alanine-substitution analysis

Western blots of (A) conditioned media and (B) cell lysates of transiently transfected HEK-293 cells expressing the described variants were performed employing anti-furin antibody MON-148. The cells were transfected with the expression vector either lacking the furin cDNA (lanes 1), or harbouring variants P686A/P687A/A688–707 (lanes 2), E684A/S685A/A688–707 (lanes 3), R683A/A688–707 (lanes 4), S682A/R683A/A688–707 (lanes 5), Q680A/S681A/A688–707 (lanes 6) and Q678A/S679A/A688–707 (lanes 7). Molecular-mass markers are indicated on the left.

Since the degree and heterogeneity of glycosylation of the molecule was expected to interfere with this analysis, a 20 amino acid linker was inserted N-terminal to Gly
's, between Arg
's (but sufficiently remote from Arg
's so as to be unlikely to influence shedding) and the postulated glycosylation sites at positions 387, 440 and 553 [28,29]. This linker was designed to contain the motif LVPRGS, a cleavage site for the endoprotease thrombin, followed by a hexa-histidine affinity tag (Figure 4A).

Both of these moieties were flanked by glycine residues in order to provide conformational flexibility and accessibility of the thrombin site. This furin molecule was termed fur/3xG-FIIa-2xG-6xH-3xG and was anticipated to yield a primary translation product 814 amino acids in length. Upon expression, a thrombin-susceptible shed furin form (termed furin-IIa/His) was expected to occur in the cell-culture supernatant. Upon exposure to thrombin in vitro, furin-IIa/His was thought to yield two furin cleavage products, the smaller of which represented the C-terminal fragment easily purified by means of its affinity epitope. The purified molecule was then subjected to MS analysis.

A HEK-293 cell clone permanently expressing fur/3xG-FIIa-2xG-6xH-3xG was established. Conditioned medium derived from this clone exhibited furin-specific functional activity (results not shown), showing that the insertion of 20 heterologous amino acids did not interfere with shedding and yielded active molecules. In Western blots employing anti-furin monoclonal antibody MON-148 directed against the catalytic domain, two bands were detected, one of which corresponded in size to the expected \( \approx 84 \text{kDa} \) shed furin-IIa/His form. The other band had a molecular mass of \( \approx 64 \text{kDa} \) (Figure 4B, lane 1). Upon incubation of the conditioned medium with thrombin the larger band disappeared, whereas the smaller band intensified further (Figure 4B, lane 2). With an anti-polihistidine antibody the conditioned medium, not exposed to thrombin yet, was shown to contain a small \( \approx 20 \text{kDa} \) fragment in addition to shed furin-IIa/His (Figure 4B, lane 3). Somewhat surprisingly, the size of the His-tagged band was larger than expected. This discrepancy can, however, be explained by the alteration of the overall charge of the molecule, the latter of which has been reported to result in altered migration in SDS/PAGE [30]. The thrombin-treated supernatant showed increased amounts of the small fragment (cleavage product 2), whereas the shed furin-IIa/His molecular species was absent (Figure 4B, lane 4). These findings strongly suggest that thrombin cleaves shed furin-IIa/His into the expected two fragments, as shown in Figure 4(A): cleavage product 1, retaining the N-terminal catalytic and homo-B domains of furin, which are indispensable for function, and cleavage product 2, N-terminally harbouring the heterologous polyhistidine tag, followed by the cysteine-rich domain and, ultimately, the shed furin C-terminus. An unexpected finding of furin-IIa/His shedding was the presence of significant amounts of furin-IIa/His-derived cleavage products in the conditioned medium prior to thrombin exposure. Thrombin treatment of the supernatant led to the complete conversion of shed furin-IIa/His into cleavage products 1 and 2, which corresponded in size exactly to those molecular forms already present prior to thrombin exposure, implying that the heterologous thrombin site, in a significant fraction of the molecules, had been cleaved by unidentified HEK-293 endoproteases. This cleavage probably occurs intracellularly, since prolonged incubation of the conditioned medium (without thrombin) did not result in any additional cleavage of shed furin-IIa/His (results not shown).

In order to demonstrate the absence of glycosylation on cleavage product 2, the thrombin-treated conditioned medium was subjected to N-glycanase F digestion. Since the electrophoretic mobility of cleavage product 2 was not altered by the treatment, this finding experimentally confirmed the absence of carbohydrates that could have impeded MS analyses (results not shown).

Conditioned medium containing thrombin-digested shed furin-IIa/His was applied to Ni-NTA–agarose and bound material was eluted by incrementally increased concentrations of imidazole. Eluted cleavage product 2 was dialysed and concentrated by Speed-Vac centrifugation. N-terminal amino acid sequence analysis confirmed the cleavage by thrombin (and by the HEK-293 surrogates since only one terminus was detectable) C-terminal to the arginine present within the LVPR–GS motif: cleavage product 2 correctly exhibited the expected N-terminus GSGGH. Since the electrophoretic mobility of cleavage product 2 was not altered by the treatment, this finding experimentally confirmed the absence of carbohydrates that could have impeded MS analyses (results not shown).

Alteration and deletion of Arg
's redirects processing to alternative 'shed' sites

Whereas the Arg
's substitution in furin variant A688–707 practically abolished shedding (Figure 1B, R683A/A688–707; Figure 3A, lane 4), its quantitative effect on shedding in the full-
Mapping of cleavage determinants of shed furin

Figure 4  Engineering of a shed furin molecule suitable for MS analysis, and determination of its C-terminus

(A) Schematic representation of the concept used to yield a shed furin variant fragment suitable for MS analysis. A stretch of 20 amino acid residues was inserted in between Ser585 and Gly586 of wild-type furin. This heterologous sequence harbours a consensus thrombin cleavage site, followed by a polyhistidine affinity tag. Upon expression, the resulting furin/IIa/His, which releases cleavage products 1 and 2 when treated with thrombin. The polyhistidine epitope, which is retained on cleavage product 2, facilitates subsequent purification by affinity chromatography.

E. Putative glycosylation sites; †, thrombin cleavage. (B) Identification of shed furin-IIa/His, and its cleavage products, in the conditioned medium of a stably transfected HEK-293 cell clone, before and after thrombin treatment. Western blots employing antibodies directed against the N-terminus of furin (lanes 1 and 2), and the polyhistidine tag (lanes 3 and 4) were performed, prior to (lanes 1 and 3) and after (lanes 2 and 4) the incubation with thrombin. Molecular-mass markers are indicated on the left.

(C) Mass spectrum of purified cleavage product 2.

Figure 5  Redirection of the shedding mechanism to an alternative site upon abolition of Arg683

Full-length furin mutations affecting either Arg683 or adjacent amino residues (as indicated) were transiently transfected in HEK-293 cells. Furin molecules in (A) conditioned media and (B) cell lysates were visualized by Western blotting using an anti-furin antibody. The cells were transfected with the parental expression vector either lacking the furin cDNA (lanes 1), or harbouring wild-type full-length furin (lanes 2), full-length furin mutants R683A (lanes 3), S682A (lanes 4), ΔR683 (lanes 5), E684V (lanes 6), R683K (lanes 7), R683E (lanes 8) and R683I (lanes 9). Shed wild-type (shed furin) and alternative (shed furin*) furin forms are indicated. Molecular-mass markers are indicated to the left.

Full-length furin molecule (lacking any internal deletions) was found to be pronounced but not as dramatic compared with wild-type furin (Figure 5A, lanes 3 and 2, respectively). Surprisingly however, R683A-derived shed furin exhibited a slightly larger molecular mass than its wild-type-furin-derived counterpart (shed furin* in Figure 5A). Similar effects were seen when Arg683 was replaced by lysine, glutamic acid or isoleucine (i.e. irrespective of charge), and when Arg683 was deleted (Figure 5A, lanes 7, 8, 9, 5, respectively). In contrast, Ser682, when replaced by an alanine in full-length furin, did not impair shedding and only the wild-type shed site was used (Figure 5A, lane 4). Interestingly, mutation E684V resulted in the simultaneous use of both the wild-type as well as the alternative shed site (Figure 5A, lane 6). Intracellular expression was quantitatively comparable for all constructs (Figure 5B). Unavailability/loss of the ‘shed’ determinant R683A thus results in the redirection of the ‘shedding’ mechanism to an alternative site in the immediate C-terminal vicinity.

DISCUSSION

Furin has repeatedly been reported to form a C-terminally truncated and, hence, naturally secreted molecular form, lacking the transmembrane domain and cytosolic tail, by a process
termed shedding [18–22]. Previous findings suggested that this cleavage site is located in the vicinity of and N-terminal to Leu$^{98}$ [18].

By intramolecular deletion of stretches of amino acid residues and subsequent individual amino acid substitution analyses, Arg$^{683}$ was identified to play a crucial role in the shedding process. Its individual deletion or alteration in otherwise wild-type furin resulted in a severe reduction of shedding, but not complete abolishment. Rather, an alternative site in the vicinity of and C-terminal to Arg$^{683}$ was used instead, albeit at a significantly reduced efficiency when compared with wild-type furin. Arginine residues potentially serving as ‘alternative’ determinants are present at positions 693, 703 and 705. Shed furin molecules derived from both, wild-type and alternative shedding, were found to be functionally active (results not shown).

Furin molecules derived from both, wild-type and alternative shedding, a potential phenomenon previously discussed for the cleavage of endothelial angiotensin-converting enzyme [31]. Since endoproteases commonly cleave C-terminal to arginine residues, Arg$^{683}$ was expected to represent the C-terminus of wild-type shed furin. Interestingly, employing MS analysis, the C-terminus was unambiguously identified to be Ser$^{685}$. Alteration of Ser$^{685}$ to alanine affected shedding neither quantitatively nor qualitatively. Similarly, alteration of other amino acids in the immediate vicinity of Arg$^{683}$ (in either direction) did not result in any effect on shedding with one exception. Substituting Glu$^{684}$ for a valine yielded less wild-type shed furin while simultaneously generating the slightly longer ‘alternative’ shed furin.

It remains to be determined where the actual intramolecular cleavage occurs. The presence of a bulky hydrophobic amino acid at the position immediately C-terminal to an endopeptidolytic cleavage site is known to frequently impair processing efficiency, as observed in this study for the E684V variant. This finding suggests that the potential cleavage directly precedes Glu$^{684}$. Subsequently, carboxypeptidyl trimming of Arg$^{683}$ may create the ultimate Ser$^{685}$ C-terminus of wild-type shed furin.

Although the shed cleavage site does not constitute a furin consensus sequence, it cannot be excluded that either furin itself or a related PC such as PC6 or PC7 [32,33] cleaves in a relaxed mode at Arg$^{683}$ instead of at its ‘appropriate’ di-basic recognition site. Relaxed stringency of furin-mediated cleavage has recently been reported with a soluble furin molecule for human factor X propeptide removal [30]. More likely, however, is that the amino acid composition suggests a mechanism obeying the rules for compatibility with aliphatic residues at the P1 position, features such as incompatibility with aliphatic residues at the P1’ position, favourable influence of short-side-chain amino acids like serine at the P3 and P2’ positions, and the necessity for an arginine directly preceding the cleavage site support the latter [34]. The presence of a hydrophobic leucine residue following Arg$^{683}$ and Arg$^{703}$ might explain why these mono-arginyl sites are not regularly used for shedding.

Rather than mediating hydrolysis of the Arg$^{683}$/Glu$^{684}$ peptide bond, an endoprotease may cleave N-terminal to Arg$^{683}$. Such a mechanism was reported for dynorphin-converting enzyme, which is involved in the maturation of peptide hormones such as the opioid peptide precursor dynorphin B-29 in the bovine pituitary gland [35]. Wide tissue distribution and cleavage at monobasic sites may suggest a more general role of dynorphin-converting enzyme for the processing of other precursor molecules [36,37].

Mapping of the cleavage site and determination of the cleavage specificity in furin described in this report may now aid the identification of the endoprotease(s) involved. The availability of a mutant molecule not subject to shedding provides a helpful tool to further elucidate intracellular routing processes of furin.

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