REVIEW ARTICLE

Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins

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Limited endoproteolysis of inactive precursor proteins at sites marked by paired or multiple basic amino acids is a widespread process by which biologically active peptides and proteins are produced within the secretory pathway in eukaryotic cells. The identification of a novel family of endoproteases homologous with bacterial subtilisins and yeast Kex2p has accelerated progress in understanding the complex mechanisms underlying the production of the bioactive materials. Seven distinct proprotein convertases of this family (furin, PC2, PC1/PC3, PC4, PACE4, PC5/PC6, LPC/PC7/PC8/SPC7) have been identified in mammalian species, some having isoforms generated via alternative splicing. The family has been shown to be responsible for conversion of precursors of peptide hormones, neuropeptides, and many other proteins into their biologically active forms. Furin, the first proprotein convertase to be identified, has been most extensively studied. It has been shown to be expressed in all tissues and cell lines examined and to be mainly localized in the *trans*-Golgi network, although some proportion of the furin molecules cycle between this compartment and the cell surface. This endoprotease is capable of cleaving precursors of a wide variety of proteins, including growth factors, serum proteins, including proteases of the blood-clotting and complement systems, matrix metalloproteinases, receptors, viral-envelope glycoproteins and bacterial exotoxins, typically at sites marked by the consensus Arg-Xaa-(Lys/Arg)-Arg sequence. The present review covers the structure and function of mammalian subtilisin/Kex2p-like proprotein convertases, focusing on furin (EC 3.4.21.85).

INTRODUCTION TO THE MAMMALIAN SUBTILISIN/Kex2p-LIKE PROPROTEIN CONVERTASES

History of studies on proprotein convertases

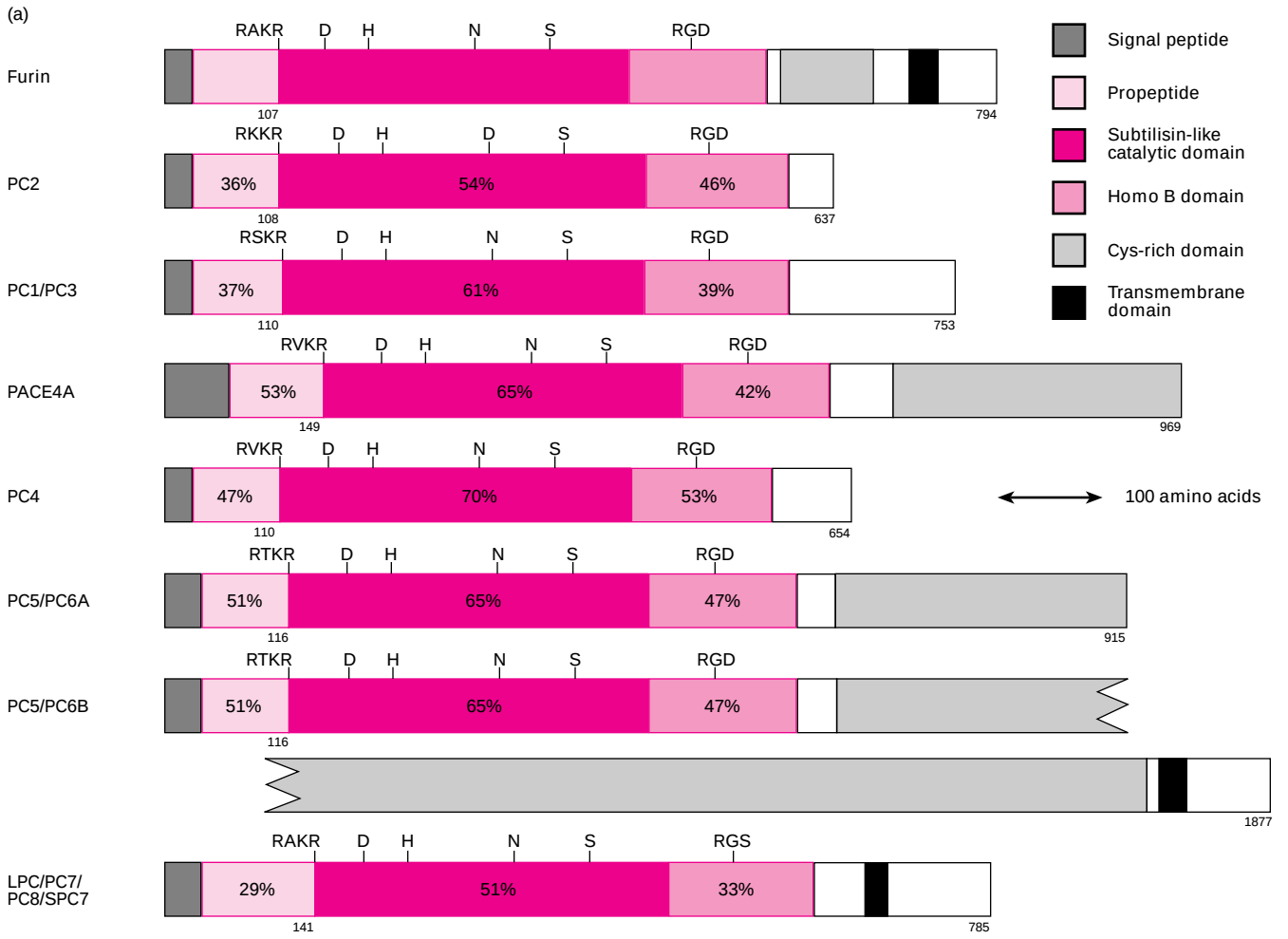
Three decades ago, two research groups independently proposed the hypothesis that peptide hormones are synthesized intracellularly as higher-molecular-mass precursors that are post-translationally converted into biologically active forms [1,2]. Steiner et al. [1] showed, by pulse–chase experiments, that the two-chain mature form of insulin is produced through cleavage of a single chain precursor, proinsulin. A later amino-acid-sequence study revealed that pairs of basic amino acids, Lys-Arg and Arg-Arg, are present at the cleavage sites of proinsulin [3]. Chrétien and Li [2] drew a similar conclusion by a comparison of the amino acid sequences of β-lipotropin (β-LPH), γ-LPH and β-melanocyte-stimulating hormone; the last two are parts of the first, and basic pairs, Lys-Lys and Lys-Arg, are present at the putative cleavage sites. Later advances in molecular biology, however, have revealed that, in organisms from yeasts to mammals, a broad spectrum of biologically active peptides and proteins are produced by cleavage of higher-molecular-mass inactive precursors at paired basic residues. These include not only most peptide hormones and neuropeptides, but also many growth factors, receptors, adhesion molecules, plasma proteases, matrix metalloproteinases, viral-envelope glycoproteins and bacterial exotoxins [4–10]. The importance of limited endoproteolysis at paired basic amino acids for peptide and protein biosynthesis stimulated researchers to attempt to identify prohormone and proprotein convertases. However, except for a few cases [11,12], earlier efforts to purify and characterize such convertases were unsuccessful, owing to the low cellular expression levels of the convertases, the presence of other cellular endoproteases with a similar sequence specificity (e.g., kallikreins, lysosomal cathepsins) and a lack of reliable *in vitro* assay systems.

Until recently, the only unequivocal example of an endoprotease involved in precursor processing was the product of the *KEX2* gene [Kex2p; also referred to as kexin (EC 3.4.21.61)] of the yeast *Saccharomyces cerevisiae*. Its physiological role was established by genetic-complementation analyses of mutant strains (*kex2*) that are defective in the production of mature α-mating factor and killer toxin, both of which are generated through intracellular cleavage of their precursors at paired basic residues [13]. Sequence analysis of the *KEX2* gene revealed that Kex2p has a catalytic domain showing sequence similarity to bacterial serine proteases belonging to the subtilisin family [13,14] (Figure 1). In 1987, Kex2p was shown to be able to cleave proalbumin *in vitro* [15]. Furthermore, Thomas et al. [16] showed that co-expression of Kex2p with a polyhormone precursor, pro-opiomelanocortin, in mammalian cells resulted in the production of mature peptides, β-LPH, γ-LPH and β-endorphin. These data made it tempting to speculate that (a) mammalian counterpart(s) of Kex2p, if present, would function as proprotein or prohormone convertase(s).

Identification of mammalian proprotein convertases

The availability of the KEX2 gene sequence accelerated research on prohormone and proprotein convertases. In 1989, Fuller et al. [17] discovered the first mammalian homologue of Kex2p by a search of sequence data bases; the *fur* gene, which had been serendipitously identified by Roebroek et al. [18,19] 3 years before by its proximity to the c-*fes*/*fps* proto-oncogene (*fur* for

Abbreviations used: LPH, lipotropin; TGN, *trans*-Golgi network; ER, endoplasmic reticulum; CK-II, casein kinase II; CHO, Chinese-hamster ovary; HA, haemagglutinin; -CH_2Cl, chloromethane ('chloromethylketone'); e*n*, embryonic day *n*; p*n*, postnatal day *n*; α_1-PIT, α_1-antitrypsin Pittsburgh; α_1-PDX, another α_1-antitrypsin variant (see the text).



(b)

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Furin       ILDDGIEKNHPDLAGNYDP  NDNRHGTRCAGEVAAVANNGVCGVGVAYN  GRGGLGSIFVWASGNGGREHDSCNCDGY  CTESHTGTSASAPLAAGIIALTLEANKNLTWRDMQHLVVQTS
PC2         *M****DYL*****Y**NA  WF**********S*A*S*NI********  ****K****Y*****D**-SY*D******  **LR*S**********A**E***VF**A*****LD*********T*L**
PC1/PC3     V*****L*W**T*IYA****  *E*K*********I*MQ***HK********  **Q*K*********************QG*N*D****  ***T*****************F**A****P***********W**
PACE4       **********RN*****P***S  *E*K**********S***SY*I**I***  **Q***********************G*Y*S****  **DG*****V***MV******A****SQ*****V***L*K**
PC4         **********D****WA****  DE**********S*T****F**A***F*  **Q***TL*I*****************LHY*N******  **DK*************M***A****PL*****L*****RA*
PC5/PC6     **********RT****MQ***A  *E*K**********T***SH*T**I*F*  **R*****V*******************SK*H*S****  **DN*****************M*******A****PF*****V**VI*R**
LPC/PC7/    VV*****V*HTIQ*IAP**S*  *G*H********I***P**SF*A*****G  **Q*F*****V*****************QHN*N**Y****  ***G*****************A*******M***M*QVRPC*****V**II*F*A
PC8/SPC7
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Figure 1 Structural comparison of mammalian subtilisin/Kex2p-like proprotein convertases

(**a**) Schematic representation of the convertases. For each region, the percentage of amino acid identity with furin is shown. The catalytic Asp, His, Ser and Asn (Asp) residues and the Arg-Gly-Asp (Ser) sequence are shown. (**b**) Alignment of the sequences around the catalytic residues of the convertases. The catalytic residues are shown in red. Asterisks represent residues identical with those of furin, and a hyphen indicates a gap introduced for alignment.

c-*fes*/*fps* **u**pstream **r**egion), coded for a protein, named furin, highly similar to Kex2p in the subtilisin-like catalytic domain and its flanking regions. At about the same time, another mammalian Kex2p homologue, PC2 (PC for **p**rohormone or **p**roprotein **c**onvertase), was identified in human insulinoma [20] and mouse pituitary [21] by the application of PCR using degenerate oligonucleotide primers complementary to conserved sequences in the catalytic domain of subtilisins, Kex2p and furin. A second related protease, named PC1 [21,22] or PC3 [23,24], was also identified in mouse pituitary [21,22] and in a mouse corticotrophic tumour cell line, AtT-20 [23,24], which produces adrenocorticotropin and β-LPH through intracellular cleavage of pro-opiomelanocortin. To date, similar PCR-based strategies have identified four additional members of the mammalian subtilisin/Kex2p family, namely PACE4 [25], PC4 [26,27], PC5/PC6 [28,29], LPC/PC7/PC8/SPC7 [30–33], except for LPC, which has been identified because of its involvement in a chromosomal translocation [30]. (Since the current nomenclature

Table 1 Nomenclature of mammalian subtilisin/Kex2p-like pro-protein convertases

Name used in this Review	Name used in the original literature	References
Furin	Furin	[18,19]
	PACE	[85]
PC2	PC2	[20,21]
PC1/PC3	PC1	[21,22]
	PC3	[23,24]
PACE4	PACE4	[25]
PC4	PC4	[26,27]
PC5/PC6	PC6	[28]
	PC5	[29]
LPC/PC7/PC8/SPC7	LPC	[30]
	PC8	[31]
	PC7	[32]
	SPC7	[33]

of mammalian proprotein convertases will no doubt confuse readers outside this research field, all names so far used for a given proprotein convertase, and references to where the names first appeared, are listed in Table 1; throughout this review all the names for a given convertase will be indicated in parallel; a better system of nomenclature would obviously be desirable in the near future.) Among the proprotein convertases, PACE4 [25,34–36], PC4 [27] and PC5/PC6 [37] have isoforms that are generated via alternative splicing of the same primary transcripts. Although not discussed in detail in this Review, many subtilisin/Kex2p counterparts have also been identified in non-mammalian eukaryotes.

General structures of mammalian proprotein convertases

Figure 1(a) schematically represents the structures of mammalian subtilisin/Kex2p-like proprotein convertases. The subtilisin-like catalytic domain that extends over $\approx$ 330 amino acids is highly conserved among the eukaryotic proprotein convertases. In particular, the active-site residues of the Asp, His and Ser catalytic triad, and an Asn residue which stabilizes the oxyanion hole in the transitional state [38], are present at corresponding positions in all members, except for PC2, where the Asn residue is replaced by an Asp (Figure 1b). The sequences flanking these residues are conserved as well (Figure 1b). In addition, the $\approx$ 140-amino-acid region following the catalytic domain, which has been variously referred to as the 'Homo B', 'P' or 'middle' domain, is also well conserved among eukaryotic convertases, including yeast Kex2p, but is absent in bacterial subtilisins. The Homo B domain is essential for catalytic activity [35,39–41] (see below). Within this domain there is a conserved Arg-Gly-Asp sequence that is reminiscent of the recognition sequence for integrins. Mutation of either of these three residues in PC1/PC3 results in loss of catalytic activity and mis-sorting of this neuroendocrine convertase towards the constitutive secretory pathway [42]. Another conserved region is the propeptide, which is autocatalytically removed through cleavage at an Arg-Xaa-Lys-Arg site during maturation of the convertases (see below). Towards the C-terminus, furin, PACE4 and PC5/PC6 A and B have a Cys-rich domain. The Cys topography is well conserved [37,43], although its role is currently unknown. Furin, PC5/PC6B and LPC/PC7/PC8/SPC7 also have a transmembrane domain near the C-terminus.

Tissue distribution and subcellular localization of mammalian proprotein convertases

The mammalian proprotein convertases can be classified into three groups on the basis of their tissue distribution. Furin [44–48], PACE4 [25,49–52], PC5/PC6 [28,29,50,51], and LPC/PC7/PC8/SPC7 [30–33] are expressed in a broad range of tissues and cell lines. In contrast, expression of PC2 [20,21,47,48,52–54] and PC1/PC3 [21–24,47,48,52–54] is limited to neuroendocrine tissues, such as pancreatic islets, pituitary, adrenal medulla and many brain areas. Expression of PC4 is highly restricted to testicular spermatogenic cells [26,27,55].

Within cells, furin [56,57] and LPC/PC7/PC8/SPC7 (J. W. M. Creemers, personal communication), both of which have a transmembrane domain (Figure 1a), are localized in the *trans*-Golgi network (TGN). Another convertase with a transmembrane domain, PC5/PC6B, is also localized in the Golgi area, although it appears not to concentrate in the TGN [58]. The neuroendocrine-specific convertases, PC2 and PC1/PC3, are mainly localized in secretory granules [59–61]. PC5/PC6A has also been reported to be localized to secretory granules [58].

EXPRESSION AND TISSUE DISTRIBUTION OF FURIN

The *fur* gene is transcribed from at least three distinct promoters; one may be a regulated promoter, while the other two have the characteristics of promoters of 'housekeeping' genes [62]. Northern-blot analysis has revealed that the major $\approx$ 4.0 kb transcript of furin is present in all tissues and cell lines examined [44,45]. Zheng et al. [48] have examined the expression of furin during rat development by *in situ* hybridization. In rat, furin mRNA is first detected in both endoderm and mesoderm at embryonic day 7 (e7) in the primitive-streak stage of embryogenesis and remains uniformly expressed until e10, when a distinctly higher level of expression is observed in the heart and liver primordia. In mid- and late-gestational stages, furin is widely expressed in the peripheral tissues. The expression pattern of furin during embryogenesis is distinct from those of other ubiquitously expressed convertases, PACE4 and PC5/PC6 [51], and from those of neuroendocrine-specific ones, PC1/PC3 and PC2 [53,54]. These observations suggest that furin plays a critical role in processing of various proproteins, such as growth-factor precursors (see below), during development. This is supported by the recent finding by Roebroek and co-workers that growth defects are first observed at e8–8.5 in furin-knockout mice which die by e11–12 (A. J. M. Roebroek, personal communication). In this context, recent observations of Takeuchi and co-workers are noteworthy [63–65]. Their results show that the expression of furin is developmentally regulated and appears to control the growth and differentiation of cells, such as pancreatic-islet cells and gastric mucous cells. In rat pancreatic islets, expression of furin is detectable at e18 and increases through the neonatal stage. The expression begins to decrease from postnatal day 10 (p10). This mode of furin expression is in contrast with that of the neuroendocrine-specific convertases, PC2 and PC1/PC3, both of which appear late during gestation and are expressed in the adult animals [53,54]. In islet-cell lines, furin expression appears to be in inverse proportion to the growth rate and insulin content of the cells. Furthermore, the islet-cell lines transfected with a furin expression vector grow faster than those transfected with a control vector, and conditioned media from furin-transfected cells cause increase in DNA synthesis of untransfected cells. Together, these observations suggest that furin may promote cell growth by elevating the production of mature mitogenic factors that require cleavage at consensus furin sites (see below).

MATURATION AND SUBCELLULAR LOCALIZATION OF FURIN

Furin is a membrane protein with a type I topology. In cells transfected with a cDNA expression plasmid or infected with a recombinant vaccinia virus, human and bovine furin are initially synthesized as 100 and 104 kDa core-glycosylated pro-furin respectively, which are rapidly converted into 94 and 98 kDa forms respectively by cleavage of the propeptide at the Arg-Ala-Lys-Arg site (residues 104–107) as schematically shown in Figure 2 [66–68]. Propeptide cleavage has been shown to be an intramolecular autocatalytic process [69,70]. Because both the pro- and mature forms are sensitive to endoglycosidase H, the autocatalytic cleavage of the propeptide appears to occur in the endoplasmic reticulum (ER) [66–68]. An interesting observation is that propeptide cleavage is a prerequisite for exit of furin molecules out of the ER [41,56,67]. Since the ER retention of pro-furin appears to be saturable [67], a putative retention protein (e.g. calnexin) may be involved in the retention. Thomas and co-workers [71] have recently demonstrated, in an elegant *in vitro* study, that propeptide cleavage is not sufficient, although it is a prerequisite, for the activation of furin. Even after its cleavage in the ER, the propeptide remains associated with the mature furin moiety and functions as a potent autoinhibitor of the endoprotease, since it can be co-immunoprecipitated with the mature moiety. Upon exposure of the latent form of furin to acidic and Ca^{2+}-containing conditions that mimic those in the TGN, the propeptide is released, generating active furin. The propeptide release at the acidic pH requires a second cleavage at the Arg-Gly-Val-Thr-Lys-Arg site (residues 70–75) in the middle of the propeptide, since mutations of either Arg residue in this sequence results in an endoprotease that cannot be activated by the acid and calcium treatment. Taken together with its TGN localization at steady state (see below) and with the data that a truncated furin construct with the ER retention signal, KDEL in one-letter code, at the C-terminus can cleave its propeptide but not substrate precursors [56,72], it is likely that furin is activated and functions mainly in the TGN.

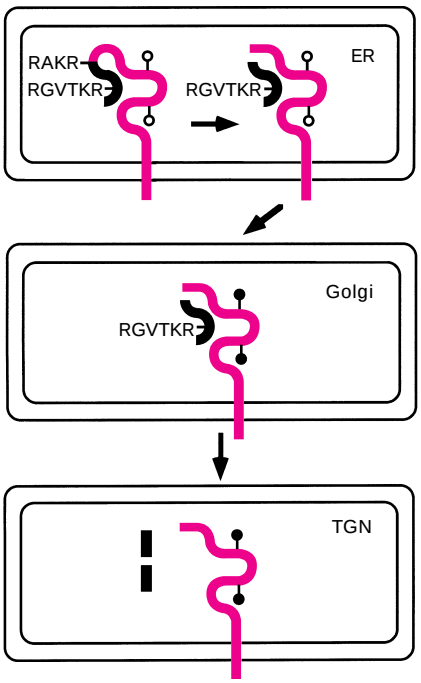

Figure 2 Schematic representation of the activation pathway of furin

The mature furin and propeptide portions are shown in red and black respectively. Open and filled 'lollipops' (♀♀) represent high-mannose-type and complex-type oligosaccharide chains respectively. The first cleavage site sequence, Arg-Ala-Lys-Arg^{107}, at the C-terminus of the propeptide and the second cleavage site sequence, Arg-Gly-Val-Thr-Lys-Arg^{75}, within the propeptide, are shown.

Immunocytochemical studies revealed that, at steady state, furin is localized in the TGN [56,57,73]. Indirect evidence, however, has suggested that a proportion of furin molecules are also present on the cell surface; a single-chain precursor of anthrax-toxin protective antigen and of diphtheria toxin, both of which have Arg-Xaa-(Lys/Arg)-Arg cleavage-site sequences (see below), can be activated to yield the two-chain mature toxin on the cell surface [74,75]. Furthermore, it has been shown that furin cycles between the TGN and cell surface [56], like another TGN membrane protein, TGN38 (for a review, see [76]). Recent studies have revealed that the cytoplasmic domain of furin is responsible for its TGN localization and recycling from the cell surface; deletion of this domain results in mislocalization of furin, and transplantation of this domain to the ectoplasmic and transmembrane domains of plasma-membrane proteins localizes the fusion proteins to the TGN [57,77–80]. Furthermore, these studies have shown that there are at least two distinct determinants in the cytoplasmic domain that contribute to the steady-state localization and trafficking of furin. One determinant is a Tyr-containing sequence, YKGL (Figure 3; residues 759–762), that serves mainly as a signal for internalization from the plasma membrane [57,79,81]. The YKGL fits the YXXØ consensus sequence (where X stands for any amino acid and Ø for an amino acid with a bulky hydrophobic side chain), which has been implicated not only in internalization of membrane proteins from the cell surface, but also in their localization to a variety of intracellular compartments, such as endosomes, lysosomes and the TGN (for a review, see [82]). Although the YXXØ motif has been shown to be involved in direct interaction with clathrin adaptor complexes, AP-1 and AP-2 [82], it is currently unknown whether the YKGL sequence of furin plays a similar role. The other signal that contributes to the TGN localization of furin is a Ser-containing acidic cluster, SDSEEDE (Figure 3; residues 773–779) [57,79–81]. This sequence fits the consensus for Ser/Thr-

Figure 3 Sequence of the cytoplasmic tail of furin

Sequences that have been proposed to be involved in localization and trafficking of furin are shown in red. Circled letters 'P' (Ⓟ) indicate the phosphate groups that may be added to the Ser residues by CK-II.

phosphorylation by casein kinase II (CK-II) [83]. Indeed, the Ser residues are phosphorylated *in vivo* [80] and *in vitro* by CK-II [80,81]. Thomas and co-workers [83] have proposed a tempting model for modulation of the furin trafficking by the phosphorylation state of the CK-II site; in the phosphorylated state furin remains at the cell surface or in endosomes, whereas removal of the phosphate by a putative furin phosphatase would enhance retrieval of the endoprotease from the cell periphery to the TGN. In support of this model, they have recently used the yeast two-hybrid system to identify a protein that interacts with the phosphorylated form of furin, but not with the dephosphorylated form (G. Thomas, personal communication). Besides the Tyr-based motif and the acidic cluster, a Leu-Ile dipeptide sequence (residues 756–757), reminiscent of the di-Leu internalization signal, and a Phe residue (residue 787) (Figure 3) have been proposed to play a role in furin trafficking (H.-D. Klenk, personal communication).

Upon reaching the cell surface or during cycling between the TGN and cell surface, furin may undergo further processing at a site between the Homo B and transmembrane domains, since a shortened, but still active, form of the endoprotease is detected in the media of cells overexpressing native furin [66,84,85]. However, it is currently unknown whether the cleavage at the cell surface is an autocatalytic process or is catalysed by other proteases.

SUBSTRATES OF FURIN

Unlike the neuroendocrine-specific expression of PC2 and PC1/PC3, furin mRNA is detected in all tissues and cell lines examined so far. This widespread expression has focused the attention of researchers on the possibility that furin may be responsible for processing of precursors for constitutively secreted proteins rather than those for peptide hormones and neuropeptides, which are stored in secretory granules and secreted in response to appropriate stimuli (regulated secretion). The enzymic activity and substrate specificity of furin have been evaluated by cellular co-expression and *in vitro* studies. Initial studies using co-expression of furin and substrate precursors in mammalian cultured cell lines have demonstrated that it is capable of cleaving precursors for β-nerve growth factor [86], von Willebrand factor [85,87], complement C3 [68], and albumin [68]. Moreover, a study in my laboratory using prorenin mutants with substitution(s) around the native Lys-Arg cleavage site demonstrated that furin preferentially recognizes the cleavage-site sequence Arg-Xaa-(Lys/Arg)-Arg [10,88]. To date, co-expression studies have shown that precursors for a wide variety of proteins with a cleavage site sequence(s) fitting the Arg-Xaa-(Lys/Arg)-Arg motif are capable of being cleaved by furin. As listed in Table 2, these include growth factors, their receptors, plasma proteins involved in the blood-clotting and complement systems, matrix metalloproteinases, viral-envelope glycoproteins and bacterial exotoxins. Besides the precursors shown in Table 2, there are others with the Arg-Xaa-(Lys/Arg)-Arg sequence that have not been demonstrated to be cleaved by furin. The cleavage sites of a few of the precursors in Table 2 do not fully fit the Arg-Xaa-(Lys/Arg)-Arg consensus sequence. My colleagues and I have therefore performed further studies on the sequence specificity of furin by co-expression of furin with a series of prorenin mutants and have delineated the following sequence rules that govern the cleavage by furin [89–91]. (i) An Arg residue is essential at the P_1 position. (ii) In addition to the P_1 Arg, at least two out of the three residues at P_2, P_4 and P_6 are required to be basic for efficient cleavage. (iii) At the P_1' position, an amino acid with a hydrophobic aliphatic side chain is not suitable. The cleavage-site specificity determined by the co-expression studies is in good agreement with that determined by *in vitro* studies using purified recombinant soluble forms of furin [74,88,92–94], although an Arg-Xaa-Xaa-Arg site is also cleaved with an $\approx$ 10-fold lower efficiency than that of Arg-Xaa-(Lys/Arg)-Arg [74,88,92]. It is noteworthy that the cleavage-site sequences of most of the precursor proteins shown in Table 2 fit these rules.

On the basis of the above rules, we have speculated that furin has subsites in the substrate-binding region that contain negatively charged amino acids that may interact with the positively charged residues of substrates, suggesting that the number of interactions between the positive and negative charges may determine the affinity of the substrates for furin [90,91]. This speculation is supported by some lines of evidence. First, Siezen and co-workers [70,95,96] have constructed a model of the three-dimensional structure of the subtilisin-like catalytic domain of

Table 2 Sequences around the cleavage site of precursor proteins

These precursors have been shown to be cleaved by furin in cellular co-expression and/or *in vitro* experiments. Basic residues are shown in red.

Precursors	Cleavage site		References
Growth Factors and Hormones	P6 P4 P2P1 ↓ P1'		
Mouse pro-β-nerve growth factor	RTHRSKR	SS	[86]
Porcine pro-brain-derived neurtrophic factor	MSMRVRR	HS	[145]
Human pro-neurotrophin-3	RTSRRKR	YA	[145]
Human pro-transfoming growth factor β1	QSSRHRR	AL	[146]
Rat pro-Müllerian inhibiting substance	GRGRAGR	SK	[147]
Human pro-insulin-like growth factor I	KPAKSAR	SV	[148]
Human pro-endothelin-1	RLRRSKR	CS	[149]
Human pro-parathyroid hormone-related peptide	SLRRLKR	AV	[150]
Human pro-parathyroid hormone	KSVKKR	SV	[151]
Receptors	P6 P4 P2P1 ↓ P1'		
Human insulin pro-receptor	RPSRKRR	SL	[99, 152]
Human hepatocyte growth factor pro-receptor	TEKRKKR	ST	[99, 100]
Human pro-LRP	TSNRHRR	QI	[153]
Human integrin α3-chain	SPQRRRR	QL	[154]
Human integrin α6-chain	HNSRKKR	EI	[154]
Plasma Proteins	P6 P4 P2P1 ↓ P1'		
Human proalbumin	RGVFRR	DA	[68, 93]
Rat complement pro-C3	PAARRRR	SV	[68]
Human pro-factor IX	ILNRPKR	YN	[155]
Human pro-factor X	TLERRKR	SV	[156]
Human pro-von Willebrand Factor	LSHRSKR	SL	[85, 87]
Human pro-protein C	KRSHLKR	DT	[157]
Matrix Metalloproteinases	P6 P4 P2P1 ↓ P1'		
Human stromelysin-3	ARNRQKR	FV	[158]
Human MT-MMP1	ANVRRKR	YA	[159]
Viral Envelope Glycoproteins	P6 P4 P2P1 ↓ P1''		
Human immunodeficiency virus gp160	VVQREKR	AV	[128, 129]
Human cytomegalovirus glycoprotein B	THNRTKR	ST	[135]
Mouse mammary tumor virus-7 superantigen	IENRKRR	ST	[160]
Avian influenza virus A hemagglutinin	SKKREKR	GL	[123, 125]
Measles virus F0	SSRRHKR	FA	[142]
Newcastle disease virus F0	GGRRQRR	FI	[104, 124]
Sindbis virus gpE2	SSGRSKR	SV	[104]
Human parainfluenza virus type 3 F0	TDPRTKR	FF	[136]
Bacterial Exotoxins	P6 P4 P2P1 ↓ P1'		
Anthrax toxin protective antigen	SNSRKKR	ST	[74, 88]
Diphtheria toxin	AGNRVRR	SV	[75]
Pseudomonas exotoxin A	TRHRQPR	GW	[104]
Shiga toxin	HASRVAR	MA	[161]
Others	P6 P4 P2P1 ↓ P1'		
Human pro-furin	AKRRTKR	DV	[69]
Rat pro-endopeptidase 3.4.24.18	QPSRPKR	SV	[162]
Mouse pro-7B2	GQRRKRR	SV	[163]

furin on the basis of the crystal structures of subtilisin BPN′ and thermitase and predicted that there are particular negatively charged amino acids in the S_1, S_2 and S_4 subsites of the substrate-binding region. Secondly, Creemers et al. have shown that mutation of the predicted negatively charged residues alters the specificity of furin for the multiple basic residues [70]. Finally, by substituting Asp residues for non-polar residues that constitute the S_1, S_2 and S_4 subsites of subtilisin BPN′, Wells and co-workers [97,98] have engineered its variant, named furilisin, that shows substantial preference for cleaving after Arg-Xaa-Lys-Arg sequences.

Although exogenous expression of furin along with precursor proteins has proved to be useful for identification of its candidate substrates, there remains a possibility that the physiological proprotein convertase for a certain precursor may not be furin, for two reasons. One is that overexpression of furin by transient transfection or by infection of recombinant vaccinia virus may cause aberrant cleavage of precursors which never occurs under physiological conditions. The other is that three other subtilisin/Kex2p-like proprotein convertases, PACE4, PC5/PC6 and LPC/PC7/PC8/SPC7, have a sequence specificity similar to that of furin and are expressed in a broad range of tissues and cell lines. However, this problem was overcome by the use of cell lines that are unable to produce functional furin; namely, LoVo, a human colon carcinoma cell line, and RPE.40, a mutant cell line derived from Chinese-hamster ovary (CHO) cells. LoVo cells were shown to produce precursor forms of the insulin receptor and hepatocyte-growth-factor receptor [99], both of which have a consensus furin cleavage site (see Table 2). My colleagues and I have since shown that furin transfection of LoVo cells restores normal processing of these pro-receptors [100]. Cloning of furin cDNAs has revealed that LoVo cells have two mutant alleles of the *fur* gene; one is a frameshift mutation in the Homo B domain, and the other is a point mutation of a conserved Trp residue at position 547 of the Homo B domain to Arg [40,41]. Further, Moehring and co-workers [101–103] isolated RPE.40 as one of mutant strains of CHO cells that exhibits resistance to *Pseudomonas* exotoxin. This group showed that it was also resistant to diphtheria toxin and some enveloped viruses. Since the bacterial toxins and envelope glycoproteins of the viruses have a consensus furin cleavage site (see Table 2), they transfected a furin expression vector into RPE.40 cells and showed that the transfected cells became as sensitive to the toxins as wild-type CHO cells and were capable of cleaving viral glycoprotein precursors [104]. Cloning of furin cDNAs and sequence analysis of the *fur* gene in RPE.40 cells has revealed that one allele has a Cys-to-Tyr mutation at position 303 (position 196 in mature furin) near the oxyanion hole Asn residue and the other has a point mutation in an intron sequence that causes a splicing defect [105]. In these furin-deficient cell lines, all but one of the exogenously expressed precursor proteins, which had been previously shown to be cleaved by furin in cellular co-expression and/or *in vitro* studies, failed to undergo cleavage. Taken together with the fact that furin mRNA is detected in all tissues and cell lines examined so far, the data using the furin-deficient cell lines demonstrates that furin is involved in the proteolytic processing of most precursor proteins with the consensus furin cleavage sequence. The only exception is the envelope glycoprotein precursor (gp160) of HIV-1. This issue is discussed below.

ENZYMIC PROPERTIES OF PURIFIED RECOMBINANT FURIN

My colleagues and I [92], and Thomas and co-workers [88], have purified a recombinant soluble form of furin that lacks the transmembrane and cytoplasmic domains and characterized its enzymic properties. In Table 3, these properties are compared with those of PC1/PC3 [106–108] and yeast Kex2p [109–111]. Furin shows its protease activity in a broad pH range between 6.0 and 8.5 with a peak at 7.0. Chelators for bivalent cations, EDTA and EGTA, strongly inhibit the convertase activity, and 1–2 mM Ca^{2+} is required for its full activity; from its three-dimensional structural model, furin is predicted to have at least two Ca^{2+}-binding sites [95]. These properties are in contrast with those of PC1/PC3, which has a relatively acidic pH optimum (pH 5.0–6.5) and requires concentrations of Ca^{2+} above 10 mM for full activity. These differences may reflect the difference in micro-environments within organelles where the proteases are active, namely the TGN for furin and secretory granules for PC1/PC3.

Table 3 Enzymic properties of furin, PC1/PC3 and Kex2p

Numbers in square brackets are the relevant references. Abbreviations: *p*CMBS, *p*-chloromercuribenzenesulphonate; DFP, di-isopropyl fluorophosphate; DTT, dithiothreitol; *p*HMB, *p*-hydroxymercuribenzoate; *p*HMBS, *p*-hydroxymercuribenzenesulphonate; *p*APMSF, *p*-aminophenylmethanesulphonyl fluoride; *p*CMB, *p*-chloromercuribenzoate.

Convertase	Optimum pH	Optimum Ca^{2+} concn. (mM)	Inhibitors (mM)‡
Furin			
[92]	7.0 (6.0–8.5)*		*p*CMBS (2.0), $HgCl_2$ (2.0), $ZnCl_2$ (2.0), $CuSO_4$ (2.0), EDTA (5.0)
[88]	7.5 (6.0–9.0)	1.0 (0.2–10)†	PMSF (20), DFP (30), DTT (1.0), $HgCl_2$ (1.0), $ZnCl_2$ (1.0), EDTA (2.0), EGTA (2.0)
PC1/PC3			
[106]	6.0 (5.0–6.5)	20 (5.0–20)	*p*CMBS (1.0), EDTA (5.0), D-Tyr-Ala-Lys-Arg-CH_2Cl (0.1), Pro-Gly-Lys-Arg-CH_2Cl (0.1)
[107]	6.0 (5.5–6.0)	50 (2.5–50)	*p*HMB (1.0), EDTA (10.0), EGTA (10.0), Lys-Arg-CH_2Cl (0.1)
[108]	6.0 (6.0–6.5)	10 (1.0–50)	*p*HMBS (1.0), EDTA (2.0), EGTA (2.0)
Kex2p			
[109,110]	6.5–9.5 (5.7–9.5)	> 0.5 (> 0.25)	Antipain (5.0), leupeptin (5.0), DFP (20), Ala-Lys-Arg-CH_2Cl (0.5)
[111]	5.5 (5.5–7.0)	> 1.0 (> 0.01)	Leupeptin (10), DFP (10), PMSF (3.3), *p*APMSF (3.3), iodoacetate (10), iodoacetamide (10), *p*CMB (1.0), $HgCl_2$ (1.0), $ZnCl_2$ (1.0), EDTA (1.0), EGTA (10), pepstatin A (10)

* A pH range with > 50% of the maximum activity is shown in parentheses.

† A range of Ca^{2+} concentration with > 50% of the maximum activity is shown in parentheses.

‡ The name and concentration (in parentheses) of inhibitors that show > 80% inhibition of the convertase activity are shown.

Although furin and other proprotein convertases are serine proteases in their primary structure, relatively high concentrations of typical serine-protease inhibitors, such as PMSF and di-isopropyl fluorophosphate, are required for inhibition of activity (Table 3). By contrast, cysteine-protease inhibitors, such as mercury-containing compounds and heavy-metal ions, strongly inhibit the activity. This may reflect the presence of a unique unpaired Cys residue near the active-site His residue [95].

We [92], Thomas and co-workers [88] and Lazure, Seidah and co-workers [112] have used a series of fluorogenic peptides to screen their effectiveness as substrates for purified furin. These studies have revealed that the Arg residues at the P_4 and P_1 positions are absolutely required for cleavage by furin. A basic

residue (Lys or Arg) at the P_2 position enhances the cleavage efficiency. These basic residue requirements are in good agreement with those determined by *in vitro* studies using protein substrates [74,88,91]. Furthermore, the study by Lazure, Seidah and co-workers [112] has shown that furin does not favour an acidic amino acid at the P_3 position.

GENETIC DISORDERS WITH A MUTATION AT THE FURIN CLEAVAGE SITE

Since furin cleavage is essential for the production of a wide variety of biologically active proteins (see Table 2), it is possible that mutation of the furin cleavage site of the precursors may result in genetic disorders. In 1978, Brennan and Carrell [113] found that a family with a circulating variant of human proalbumin, named proalbumin Christchurch, had a mutation of the P_1 Arg residue to Gln (Table 4). To date, many proalbumin variants with a mutation of the Arg residue at the P_1 or P_2 position to a non-basic residue have been reported; several of them are shown in Table 4(A). Brennan and I have shown that neither of the examined proalbumin variants is cleaved by furin *in vitro* [93]. The only known functional difference of these proalbumin variants from mature serum albumin is their decreased affinity for binding Cu^{2+}. Since a homozygous individual with one of these variants has been reported [114], such mutations may not be lethal. It is interesting to note that proalbumin Bleinheim, which has a mutation of the P_1' Asp residue to Val, is not cleaved by furin [93,115]; this is compatible with one of our proposed rules that a hydrophobic aliphatic residue at the P_1' position is not suitable for precursor cleavage by furin (see above). In this context, a recent report by Brennan et al. on a fibrinogen variant, named fibrinogen Canterbury [116], is also worthy of note. They have found that a heterozygous patient with prolonged thrombin time and a mild bleeding tendency has a mutation of Val^{20} to Asp near the fibrinopeptide A cleavage site of the fibrinogen A α-chain. This mutation changes the normal Arg^{16}-Gly-Pro-Arg-Val^{20} sequence to Arg-Gly-Pro-Arg-Asp, creating a potential furin cleavage site at Arg^{19} (Table 4A). This aberrant intracellular cleavage probably by furin gives rise to a three-residue truncated form of the α-chain without cleavage by thrombin.

In 1989, Bentley et al. [117] reported that a severe form of haemophilia B patient, named haemophilia B Oxford-3, had a mutation of the P_4 Arg residue to Gln of pro-Factor IX. To date, there have been many reports of haemophilia B cases with mutations of the P_4, P_2 or P_1 basic residue of pro-Factor IX (for a review, see [118]) (Table 4A). In 1988, two members of a Japanese family with extreme insulin resistance were reported to have a mutation of the P_1 Arg residue to Ser at the cleavage site of insulin pro-receptor (Table 4A), resulting in the production of the unprocessed pro-receptor [119,120].

Thus mutations at the furin cleavage site of a number of precursor proteins are responsible for a variety of genetic disorders. Future analysis of other genetic disorders may reveal similar mutations.

Table 4 Sequences around the cleavage site of precursor proteins and their variants

(A) Cleavage-site sequences of proalbumin, pro-Factor IX, pro-fibrinogen A α-chain and insulin pro-receptor, and their variants observed in patients with a genetic disorder. Basic residues are shown in red and substituted residues are underlined. (B) Cleavage-site sequences of HAs of virulent and avirulent influenza viruses and protein F0 of virulent and avirulent Newcastle-disease viruses. Basic residues are shown in red.

A

Precursor Protein	Cleavage Site		
	P_6 P_4 P_2P_1	P_1'	
Proalbumin			
wild type	RGVFRR	DA	
Lille	RGVFHR	DA	
Kaikoula	RGVFCR	DA	
Christchurch	RGVFRQ	DA	
Jaffna	RGVFRL	DA	
Takefu	RGVFRP	DA	
Bleinheim	RGVFRR	VA	
Pro-factor IX			
Wild type	ILNRPKR	YN	
Oxford-3	ILNQPKR	YN	
Boxtel	ILNWPKR	YN	
Bendorf	ILNLPKR	YN	
Seattle E	ILNRPNR	YN	
Cambridge	ILNRPKS	YN	
Insulin pro-receptor			
Wild type	RPSRKRR	SL	
Japan	RPSRKRS	SL	
Fibrinogen A α-chain			
Wild type	GGVRGPR	VV	
Canterbury	GGVRGPR	DV	

B

Virus (Strain)	Cleavage Site		Infection Mode
	P_6 P_4 P_2P_1	P_1'	
Influenza virus HA			
A/Puerto Rico/8/34	IPSIQSR	GL	Local
A/Japan/305/57	VPQIQSR	GL	Local
A/Memphis/102/72	VPEKQTR	GL	Local
A/Duck/Ukraine/1/63	VPEKETR	GL	Local
A/Seal/Mass/1/80	PENPKTR	GL	Local
A/FPV/Rostock/34	SKKREKR	GL	Systemic
Newcastle disease virus F0			
Avirulent	GGGKQGR	LI	Local
Virulent	GGRRQRR	FI	Systemic

FURIN AND VIRAL INFECTION

Proteolytic activation of envelope glycoproteins is necessary for the entry of viruses into host cells and, hence, for their ability to undergo multiple replication cycles. In some cases, it has also been shown that the cleavability of the envelope glycoproteins is an important determinant for viral pathogenicity. The haemagglutinins (HAs) of mammalian influenza viruses and avirulent avian-influenza viruses, which cause local infection, are susceptible to proteolytic cleavage only in limited cell types, such as those of the respiratory and alimentary tracts. In contrast, those of virulent avian-influenza viruses, which cause systemic infection, are cleaved in a broad range of different host cells (for a review, see [7]). Similarly, avirulent and virulent Newcastle-disease viruses cause local and systemic infections respectively. Nucleotide-sequence analysis suggested a relationship between the viral pathogenicity and the cleavage-site sequence of envelope-glycoprotein precursors. HAs of all mammalian and avirulent avian-influenza viruses and the F0 protein of avirulent Newcastle-disease viruses have a single Arg residue at the cleavage site, with the common denominator $(Gln/Glu)^{P_3}$-Xaa-Arg^{P_1} (Table 4B). It has been shown that an endoprotease, named tryptase Clara, which is specifically associated with bronchiolar

epithelial cells, is involved in this type of the precursor cleavage ([121]; reviewed in [122]). This is consistent with the fact that the avirulent viruses cause an infection localized to respiratory and alimentary tracts. In contrast, the virulent viruses have glycoprotein precursors that are cleaved at a site marked by the consensus sequence (Lys/Arg)^{P_5}-Arg-X-(Lys/Arg)-Arg^{P_1} (Table 4B), which completely fits the consensus sequence for cleavage by furin. *In vitro* experiments using purified furin and co-expression experiments using LoVo and RPE.40 cells as the host cells have shown that furin is indeed involved in the cleavage of the glycoprotein precursors of virulent viruses [123–127]. Thus the ubiquitous expression of furin can account for systemic infections by the virulent viruses.

On the other hand, there is currently a debate on proprotein convertase(s) that are physiologically involved in cleavage and activation of HIV-1 gp160. Earlier cellular co-expression and *in vitro* studies have shown that furin can cleave gp160 to yield gp120 and gp41. This, taken together with furin's expression in $CD4^+$ cell lines, has led to the proposal that furin is the protease activating gp160 [128,129]. For the following two reasons, however, other proteases may also be involved physiologically in gp160 cleavage. One is the study by my colleagues and I showing that, even in the furin-deficient cell line LoVo, gp160 is cleaved to yield gp120 and gp41 [126], whereas the F_0 protein of virulent Newcastle-disease virus or HA of virulent influenza virus is not cleaved [126,127]. The other is that other subtilisin/Kex2p-like convertases can also cleave gp160 [130,131]. On the basis of expression in $CD4^+$ cell lines and T lymphocytes, and cleavage activity toward gp160, Seidah and co-workers [132,133] have recently suggested that furin, PC5/PC6 and LPC/PC7/PC8/SPC7 are the major gp160-converting enzymes in T lymphocytes. On the other hand, and on the basis of similar criteria, Franzusoff and co-workers [134], and Garten, Klenk and co-workers [131], have suggested that furin and PC5/PC6, or furin and LPC/-PC7/PC8/SPC7 respectively, are the major gp160 convertases.

ATTEMPTS TO DEVELOP INHIBITORS FOR FURIN AND OTHER SUBTILISIN/Kex2p-LIKE PROPROTEIN CONVERTASES

Attempts have been made to develop compounds that specifically perturb the activity of furin, since such compounds may inhibit infection by viruses, such as HIV, through inhibiting maturation of their envelope glycoprotein. Garten, Klenk and co-workers [123] have shown that acylated peptidyl chloromethanes (-CH_2Cl; 'chloromethylketones') containing a consensus furin cleavage sequence, such as decanoyl-Arg-Glu-Lys-Arg-CH_2Cl, inhibit cleavage of influenza-virus HA by furin *in vitro* at micromolar concentrations through covalently modifying the substrate-binding site of the convertase. They have subsequently shown that, when applied to cells infected with viruses, these peptides block the cleavage of envelope glycoprotein precursors, such as influenza HA, HIV gp160, cytomegalovirus glycoprotein B and parainfluenza-virus glycoprotein F_0, thereby inhibiting formation of infectious viruses [123,128,135,136]. Although these peptidyl-CH_2Cl species are very useful for studies on proprotein convertases, they appear to be ineffective for antiviral therapy. One reason is that they are unable to abolish completely cleavage of these glycoprotein precursors, possibly due to their low efficiency of penetration into cells and the unstable nature of the -CH_2Cl group. Secondly, they are relatively cytotoxic, possibly due to their irreversible mechanism of inhibition. In order to circumvent these disadvantages, Angliker [137] has designed reversible peptide inhibitors, in which the -NH- group of the scissile P_1–P_1' bond has been replaced with a methylene group or a methylene group has been inserted between the -CO- and -NH- of the scissile bond, and has found that these peptides inhibits furin *in vitro* with K_i values in the nanomolar range. On the other hand, Lazure, Seidah and co-workers have designed another type of peptide that has an unnatural amino acid at the P_1' position and shown that it inhibits furin and PC1/PC3 in the micromolar range *in vitro* [138,139]. However, no data are currently available for the effects of these peptide inhibitors on the cleavage of viral-envelope glycoprotein precursors or viral infection.

Protein-based furin inhibitors have been also developed, since tissue- or cell-type-specific expression of these inhibitors controlled by a characterized promoter could be therapeutically valuable. In late 1980s, Brennan and co-workers showed that a variant of α_1-antitrypsin, named α_1-antitrypsin Pittsburgh (α_1-PIT), that has a replacement of the reactive-site Met residue by Arg, inhibited *in vitro* the conversion of proalbumin by Kex2p [15] and by a Kex2p-like enzyme in rat liver, probably furin [11]. Ikehara and co-workers then showed that α_1-PIT transfected into rat hepatocytes inhibited intracellular processing of proalbumin and pro-complement component C3 [140]. Thomas and co-workers have recently constructed another α_1-antitrypsin variant (α1-PDX) in which the reactive-centre Ala^{P_4}-Ile-Pro-Met^{P_1} sequence has been replaced by Arg-Ile-Pro-Arg [141]. This variant inhibits furin *in vitro* with a $K_{0.5}$ of 30 ng/ml ($\approx$ 0.6 nM), which is three orders of magnitude lower than that of α_1-PIT. Furthermore, when expressed in cells, α_1-PDX is able to block completely the cleavage of HIV gp160 and the production of infectious virus [141]. Since α_1-PDX has been shown to inhibit cleavage of other viral-envelope glycoproteins [142,143], this α_1-antitrypsin variant may pave the way for antiviral gene therapy, although further studies are required. On the other hand, Lu et al. [144] have found that a variant of the ovomucoid third domain with a replacement of the reactive-centre Ala^{P_4}-Cys-Thr-Leu^{P_1} sequence with Arg-Cys-Lys-Arg inhibits furin *in vitro* with a K_i in the submicromolar range.

SUMMARY AND FUTURE PROSPECTS

The discovery of a novel family of mammalian subtilisin/Kex2p-like proprotein convertases has led to a more detailed understanding of the molecular basis of maturation of biologically active peptides and proteins. Of these convertases, furin has been shown to be expressed ubiquitously and to be the major enzyme involved in the cleavage of a wide variety of precursor proteins at sites marked predominantly by the Arg-Xaa-(Lys/Arg)-Arg sequence within the secretory pathway. Furin functions mainly in the TGN and is itself synthesized as a precursor that is autocatalytically activated in the secretory pathway. By virtue of the localization and retrieval signals within its cytoplasmic tail, furin cycles between the TGN and the cell surface, where it is involved in the activation of bacterial exotoxins.

However, many questions remain to be answered and will surely be the focus of research over the next few years. First, although there is no doubt as to the role of furin in proprotein processing in most, if not all, cells, it is unclear to what extent other convertases with a broad spectrum of tissue and cell expression and with a sequence specificity similar to that of furin, namely PACE4, PC5/PC6, and LPC/PC7/PC8/SPC7, share this role with furin. Secondly, the physiological role of cell-surface and secreted furin remains unknown. Thirdly, although signals within the cytoplasmic tail have been shown to play important roles in the localization and recycling of furin, proteins that interact with or modify these signals have not been identified. In connection with antiviral therapy, another important area of future research will be the development of specific inhibitors for furin and related endoproteases together with determination of

their precise three-dimensional structure by X-ray crystallography. Tissue- or cell-type-specific expression of protein-based inhibitors appears to be a promissory approach for antiviral gene therapy.

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