The EMBO Journal Vol.16 No.7 pp.1508–1518, 1997

Activation of the furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage

Eric D.Anderson[1], Judy K.VanSlyke, Craig D.Thulin[2], François Jean and Gary Thomas[1,3]

Vollum Institute and [1]Department of Cell and Developmental Biology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201-3098, USA

[2]Present address: Department of Zoology, Brigham Young University, Provo, UT 84601, USA

[3]Corresponding author

E.D.Anderson and J.K.VanSlyke contributed equally to these studies

Activation of furin requires autoproteolytic cleavage of its 83-amino acid propeptide at the consensus furin site, Arg-Thr-Lys-Arg107$^{\downarrow}$. This RER-localized cleavage is necessary, but not sufficient, for enzyme activation. Rather, full activation of furin requires exposure to, and correct routing within, the TGN/endosomal system. Here, we identify the steps in addition to the initial propeptide cleavage necessary for activation of furin. Exposure of membrane preparations containing an inactive RER-localized soluble furin construct to either: (i) an acidic and calcium-containing environment characteristic of the TGN; or (ii) mild trypsinization at neutral pH, resulted in the activation of the endoprotease. Taken together, these results suggest that the pH drop facilitates the removal of a furin inhibitor. Consistent with these findings, following cleavage in the RER, the furin propeptide remains associated with the enzyme and functions as a potent inhibitor of the endoprotease. Co-immunoprecipitation studies coupled with analysis by mass spectrometry show that release of the propeptide at acidic pH, and hence activation of furin, requires a second cleavage within the autoinhibitory domain at a site containing a P6 arginine (-Arg70-Gly-Val-Thr-Lys-Arg75$^{\downarrow}$-). The significance of this cleavage in regulating the compartment-specific activation of furin, and the relationship of the furin activation pathway to those of other serine endoproteases are discussed.

Keywords: activation/endoplasmic reticulum/furin/propeptide/trans-Golgi network

Introduction

Biosynthesis of proteins destined for residence within, or routing through, the secretory pathway requires an orchestrated series of events, including one or more proteolytic cleavages to yield the mature and functional molecule. The primary translation product for many bioactive proteins (e.g. growth modulators, receptors, peptide hormones and proteinases) contains, in addition to the active region, a propeptide, that is typically an N-terminal extension of the mature protein. The functions of propeptides appear to be manifold. In the case of several precursors of growth modulators (e.g. nerve growth factor and amphiregulin) the propeptides are required for stability or secretion of the active factor (Suter *et al.*, 1991; Thorne and Plowman, 1994), whereas the propeptide of transforming growth factor-β participates in formation of latent complexes (Miyazono *et al.*, 1988). The propeptide of the yeast enzyme carboxypeptidase Y is required for the receptor-mediated sorting to the vacuole (Valls *et al.*, 1990). Finally, propeptides are important for the activation of all classifications of proteinases (i.e. serine-, aspartyl-, cysteinyl- and metallo-proteinases; reviewed in Baker *et al.*, 1993).

Perhaps the most thoroughly investigated examples of propeptide-mediated proteinase activation are those of the bacterial serine proteinases, subtilisin and α-lytic protease. The N-terminal propeptides of both enzymes are required for the correct folding of their catalytic domains and can facilitate the refolding of denatured enzyme *in vitro* (Power *et al.*, 1986; Ikemura and Inouye, 1988; Silen *et al.*, 1989; Zhu *et al.*, 1989). Following translation of the nascent chain into the periplasmic space and folding of the zymogen, the N-terminal propeptides of both bacterial endoproteases are cleaved by an intramolecular reaction (Power *et al.*, 1986; Silen *et al.*, 1989). The propeptides remain associated with the catalytic domains through non-covalent interactions and act as potent autoinhibitors (Baker *et al.*, 1992; Li *et al.*, 1995). Structural and biochemical analyses have shown that the subtilisin propeptide binds to the enzyme primarily through multiple non-polar interactions, with the C-terminus extending into the active site of the enzyme, thus acting as a competitive inhibitor (Li and Inouye, 1994; Bryan *et al.*, 1995; Gallagher *et al.*, 1995; Li *et al.*, 1995). When subtilisin E propeptide is degraded, by an as yet uncharacterized pathway, the enzyme is free to act on substrates *in trans* (Ikemura and Inouye, 1988).

The bacterial subtilisins are evolutionarily related to the eukaryotic proprotein convertases, a family of calcium-dependent serine endoproteases. The convertases catalyze the proteolytic maturation, and hence activation, of precursor proteins within the secretory pathway by cleavage at oligo-basic amino acid sequences (Steiner *et al.*, 1992; Smeekens, 1993). Members of the proprotein convertase family include the yeast convertase Kex2p, which catalyzes the activation of α-mating pheromone, and a number of Kex2p homologs expressed in metazoa including furin, PC1/3, PC2, PC4, PC5/6, LPC/PC7/8 (hereafter termed PC7) and PACE-4 (Bruzzaniti *et al.*, 1996; Meerabux *et al.*, 1996; Seidah *et al.*, 1996; for reviews, see Steiner *et al.*, 1992 and Smeekens, 1993).

Like the bacterial subtilisins, the proprotein convertases are synthesized as inactive precursors that require proteo-

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lytic cleavage of their N-terminal propeptides. This was first established for furin, a type I membrane protein concentrated in the TGN/endosomal system (Molloy *et al.*, 1994; Jones *et al.*, 1995; Schafer *et al.*, 1995; Takahashi *et al.*, 1995; Voorhees *et al.*, 1995) that cleaves a large number of proprotein molecules at the consensus furin site -Arg-X-Lys/Arg-Arg- in both the biosynthetic and endocytic pathways (for reviews, see Bresnahan *et al.*, 1993; Van de Ven *et al.*, 1993). The furin zymogen undergoes autocatalytic cleavage of its 83-amino acid N-terminal propeptide at the C-terminal side of the consensus furin site -Arg-Thr-Lys-Arg107$^{\downarrow}$ soon after deposition of the molecule into the RER ($t_{\frac{1}{2}}$ <10 min) (Leduc *et al.*, 1992; Molloy *et al.*, 1994; Vey *et al.*, 1994; Creemers *et al.*, 1995). Not only is the RER-localized propeptide cleavage a necessary step for activation of furin, it is also required for export of the protein from this compartment (Molloy *et al.*, 1994; Creemers *et al.*, 1995). However, this cleavage alone is not sufficient for enzymatic activation of furin. Rather, furin activation also requires exposure of the endoprotease to post-RER compartments (Molloy *et al.*, 1994; Vey *et al.*, 1994). For example, a furin construct unable to be exported from the RER undergoes autoproteolytic cleavage of its propeptide, but remains proteolytically inactive against substrates *in trans* (Molloy *et al.*, 1994).

To begin identification of the additional steps comprising the furin activation pathway, we have developed a simple *in vitro* system to study this process under defined conditions. Here, using this approach, we report the discovery of an ordered series of steps required for the activation of furin. The relationship of these steps to the compartment-dependent activation of furin in the secretory pathway is discussed.

Results

To determine the requirements for the activation of furin, a series of epitope-tagged furin constructs was used (Figure 1). Introduction of the FLAG epitope tag immediately C-terminal to the propeptide cleavage site permits the use of immunologic methods to monitor propeptide cleavage and correlation of this event with enzyme activity. Importantly, the FLAG tag at this position does not affect propeptide cleavage or enzyme activity measurably *in vivo* (Molloy *et al.*, 1994). By employing two FLAG peptide-specific antibodies, mAb M1 and mAb M2, the zymogenic form of furin (which reacts with mAb M2 only) can be distinguished from the mature forms (i.e. propeptide-cleaved) of furin generated by autoproteolytic cleavage C-terminal to Arg107 (reacts with both mAb M1 and mAb M2).

In initial studies, the importance of propeptide cleavage in furin activation was determined (Figure 2). Replicate plates of BSC-40 cells were either mock-infected (lane 1), or infected with wild-type vaccinia virus (lane 2), with vaccinia recombinants expressing the TGN localized fur/f (lane 3), or one of two furin constructs that are concentrated in the RER; a truncated soluble form of furin containing the RER retrieval signal -Lys-Asp-Glu-Leu$_{COOH}$, fur/fΔtc-k (lane 4), or the active site mutant fur/fD$_{153}$N (lane 5). Analysis of crude membrane preparations by Western blot (Figure 2A and B) showed that both fur/f and fur/fΔtc-k had undergone the autoproteolytic propeptide cleavage (both recognized by mAb M2 and mAb M1) whereas fur/fD$_{153}$N failed to undergo this step (reactive with mAb M2 only). Each of the samples was then assayed for furin enzymatic activity using the Q1 internally quenched peptide substrate (Figure 2C). Only the membrane sample from cells expressing fur/f was capable of efficiently cleaving the substrate.

The inability of the RER-localized fur/fΔtc-k to cleave *in vitro* the synthetic furin substrate, under conditions in which the TGN-localized fur/f construct could, is in agreement with our previous work demonstrating that propeptide cleavage is necessary but not sufficient for furin activation (Molloy *et al.*, 1994). Additional factors, including cellular calcium stores, are also important for furin activation. The concentration of available calcium in the RER versus the TGN is not firmly established (see Discussion); however, one measured difference between these compartments is pH. The pH of the RER is neutral (Mellman *et al.*, 1986) whereas that of the TGN is ~6.2 (Seksek *et al.*, 1995). To determine directly whether calcium and/or the acidification of the TGN contribute to furin activation, fur/fΔtc-k in crude membrane preparations was measured with or without pre-incubation in the presence or absence of 5 mM calcium under neutral (pH 7.5) or acidic (pH 6.0) conditions (Figure 3). Consistent with the data in Figure 2, fur/fΔtc-k failed to be activated at neutral pH, irrespective of the presence of calcium. By contrast, exposure of fur/fΔtc-k to a TGN-like acidic and calcium-containing environment resulted in a striking increase in enzyme activity. Importantly, the activation of fur/fΔtc-k required a pre-incubation under these conditions (compare lanes 1 and 6 with lane 5).

The pH optimum for activation of fur/fΔtc-k was determined using a range of buffers (Figure 4A). Maximal activation occurred at pH 6.0. A time-course of fur/fΔtc-k activation at pH 6.0 was next determined (Figure 4B). Under these conditions enzyme activity increased linearly for ~180 min.

The results in Figures 2 and 3 demonstrated a requirement for calcium and an acidic pH for the activation of furin following the autoproteolytic cleavage of its 83-amino acid propeptide. However, because furin undergoes an autoproteolytic cleavage of its N-terminal propeptide in the RER, the endoprotease must be intrinsically active. These findings suggest that the acidic environment of the TGN facilitates the removal of a furin inhibitor. To examine this possibility, a crude membrane fraction was prepared from BSC-40 cells expressing fur/fΔtc-k. An aliquot of the membrane sample was then subjected to limited digestion with trypsin to facilitate degradation of the furin inhibitor, followed by an assay for furin activity (Figure 5). A sequential treatment of the sample with trypsin followed by addition of soybean trypsin inhibitor (to block trypsin selectively) resulted in a large increase in furin activity compared with control samples (compare column 3 with columns 1 and 2). These results are consistent with the hypothesis that fur/fΔtc-k is inactive at neutral pH because of its association with a trypsin-labile inhibitor.

Because the N-terminal propeptides of the structurally related bacterial subtilisins are potent autoinhibitors, we examined the ability of the furin propeptide to inhibit

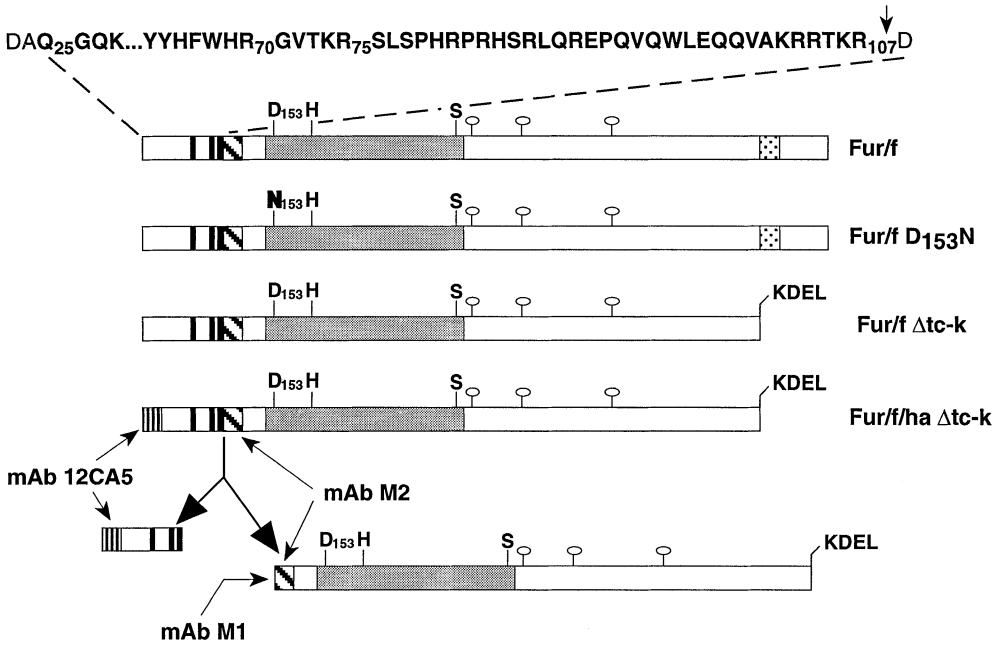

Fig. 1. Schematic of furin constructs. Recombinant furin constructs were generated using loop-in or site-directed mutagenesis. In construct fur/fD$_{153}$N the aspartic acid residue of the catalytic triad has been changed to an asparagine, resulting in inactivation of the protease. The truncated furin constructs fur/fΔtc-k and fur/f/haΔtc-k lack the sequences encoding the furin transmembrane domain (stippled box) and cytoplasmic tail, and have the ER-retention/recycling signal KDEL at their C-termini. Fur/f, fur/fD$_{153}$N and fur/fΔtc-k all have the FLAG epitope tag inserted directly after the proregion cleavage site (diagonal bars), so the N-terminus of the FLAG sequence is exposed upon autoproteolytic cleavage. The anti-FLAG mAb M2 can recognize either the blocked or exposed form of the FLAG epitope. The anti-FLAG mAb M1 can only recognize the FLAG epitope if it has a free N-terminus, and therefore is only able to detect the mature form of furin. In fur/f/haΔtc-k the HA epitope has been inserted directly after the signal sequence cleavage site (vertical bars). This epitope is recognized by the monoclonal antibody 12CA5. The subtilisin-like catalytic domain of furin is denoted by a shaded box. The residues of the catalytic triad are indicated (Asp, His, Ser). Potential glycosylation sides are denoted by 'lollipops'. Pairs of basic amino acids in the proregion are indicated by thick vertical bars.

furin's activity *in vitro*. A GST fusion protein containing residues encoding the entire furin propeptide (Gln25→Arg107), GSTpro, was prepared. Increasing amounts of the purified fusion protein were then pre-incubated for 1 h with furin enzyme *in vitro* prior to addition of an AMC substrate (Figure 6). Furin activity was inhibited with near-stoichiometric quantities of added propeptide. The inhibitory effect was specific for sequences within the furin propeptide since GST showed no inhibitory activity.

Together, the results in Figures 2 and 6 argue that the furin propeptide functions as a tightly bound autoinhibitor and suggest that removal of this fragment requires exposure of the complex to an acidic pH. Therefore, to monitor the fate of the furin propeptide during enzyme activation, a dual epitope tag construct was generated (Figure 1). In this construct, fur/f/haΔtc-k, the hemaglutinin (HA) epitope tag was inserted by loop-in mutagenesis C-terminal to the predicted signal peptidase cleavage site (Ala24$^{\downarrow}$Gln25; see Figure 1). Western blot analysis and time-course of activation studies showed that fur/f/haΔtc-k underwent correct propeptide cleavage and was activated with similar kinetics as fur/fΔtc-k (data not shown). To show that the HA tag did not affect the furin inhibitory properties of the propeptide, a second GST fusion protein, GSTpro/ha, was constructed containing the HA-tagged furin propeptide. Enzyme inhibition studies showed that GSTpro/ha attenuated furin activity at concentrations very similar to GSTpro (Figure 6).

The potent autoinhibitory properties of the furin propeptide suggest that the lack of activity of fur/fΔtc-k when incubated at neutral pH may be the result of a tight-binding interaction between the mature enzyme and the cleaved propeptide domain. To demonstrate directly the association of the furin propeptide with the enzyme *in vivo*, a co-immunoprecipitation experiment was performed (Figure 7). Replicate samples of a crude membrane preparation from BSC-40 cells expressing fur/f/haΔtc-k were incubated at either pH 7.5 or pH 6.0 and the furin construct was immunoprecipitated with mAb M1 either immediately or following preincubation in either of the two buffers. Analysis of the mAb M1 immunoprecipitates by Western blot using the mAb 12CA5 showed that the HA-tagged propeptide is associated with the mature enzyme (Figure 7A). At pH 7.5, the propeptide–enzyme complex remained stable during an extended incubation (Figure 7A, lane 2). By contrast, incubation of the complex at pH 6.0 resulted in release of propeptide from the endoprotease (Figure 7A, lane 4). The loss of co-immunoprecipitating propeptide was coincident with a marked increase in furin activity (Figure 7B). Interestingly, Western blot analysis of the whole extract showed that the propeptide underwent a second proteolytic cleavage, generating an ~6 kDa HA-tagged peptide, that was coincident with its release from the enzyme and furin activation (Figure 7C).

Identification of the ~6 kDa HA-tagged propeptide fragment was accomplished using mass spectrometry (MS) (Figure 8). A crude membrane sample prepared from BSC-40 cells expressing fur/f/haΔtc-k was incubated at pH 6.0 and the extract fractionated by reversed-phase HPLC. The column fractions containing the HA-tagged ~6 kDa band (determined by Western blot) were then subjected to MALDI–TOF MS and electrospray LC/MS.

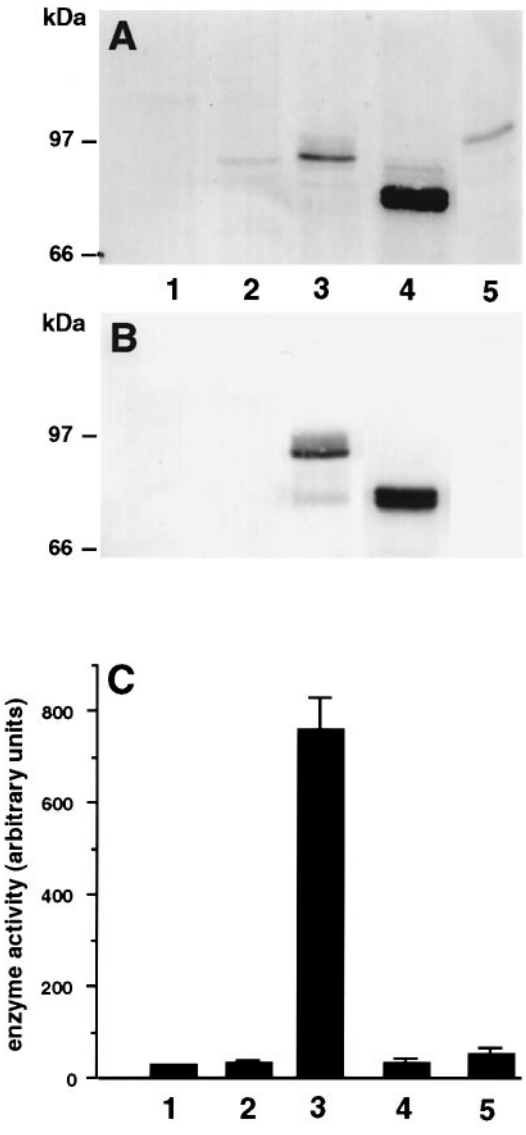

Fig. 2. Expression, immunoreactivity and *in vitro* activity of furin constructs. (**A** and **B**) Western blot analyses of equivalent amounts of crude membrane samples prepared from BSC-40 cells either mock-infected (lane 1) or infected with VV:WT (lane 2), VV:hFur/f (lane 3), VV:hFur/fΔtc-k (lane 4) or VV:hFur/fD_{153}N, (lane 5). Flag-tagged furin constructs were detected using either mAb M2 (A) or mAb M1 (B, requires propeptide cleavage). The apparent differential expression of the various constructs results in large part from their proteolytic shedding (fur/f), stable accumulation (fur/fΔtc-k) or degradation (fur/fD153N). (**C**) Proteolytic activity from the same membrane preparations were determined with the Q1 substrate (see Materials and methods). Each reading represents the average of two samples assayed in duplicate. Furin activity is shown in arbitrary units of fluorescence. Bars indicate standard deviations.

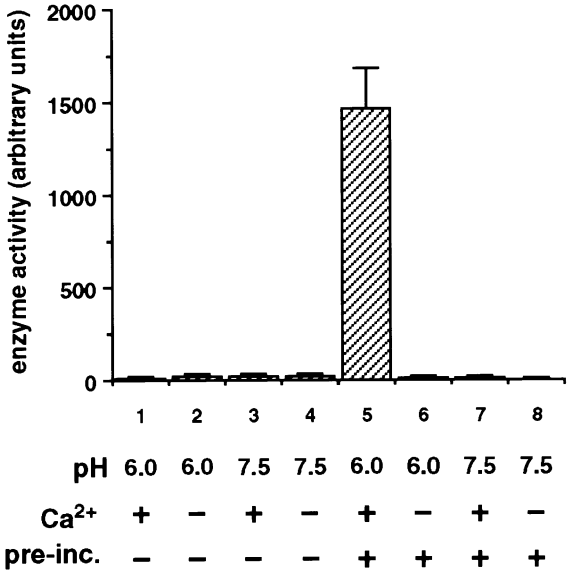

Fig. 3. *In vitro* activation of ER-retained furin by low pH and calcium. Crude membrane preparations of BSC-40 cells infected with VV:hFur/fΔtc-k were resuspendend in Bis–Tris buffer at pH 6.0 or 7.5, with or without 5 mM $CaCl_2$ as indicated. These samples were then pre-incubated at 30°C for 0 h (columns 1–4) or for 3 h (columns 5–8). Following pre-incubation, replicate aliquots of each sample was assayed for proteolytic activity using the Q1 substrate. Each reading represents two separate determinations performed in duplicate. Bars indicate standard deviations.

Analysis by MALDI showed that the ~6 kDa HA-tagged band was composed of three species with masses of: (i) 7032 ± 2.7 a.m.u.; (ii) 6878.3 ± 4.1 a.m.u.; and (iii) 6753.9 ± 3.3 a.m.u. Analysis by LC/MS allowed refinement of the mass measurements for each species to 7035.5 a.m.u., 6880.0 a.m.u. and 6752.0 a.m.u., respectively. Using mass spectrometric methods, we showed that the 83-amino acid propeptide was cleaved to generate three distinct N-terminal fragments with masses of 7036, 6880 and 6752 a.m.u. These masses are 17 a.m.u. less than the predicted HA-tagged-Gln25→Arg75, HA-tagged-Gln25→Lys74 and HA-tagged-Gln25→Thr73, respectively. However, because N-terminal glutamine residues readily cyclize, generating products that are 17 a.m.u. smaller than predicted (Krishna and Wold, 1993), we conclude that the N-terminus of profurin initiates at Gln25, which becomes cyclized to pyroglutamic acid (pGlu). Thus, these peptides were identified as: (i) HA-tagged-pGlu25→Arg75; (ii) HA-tagged-pGlu25→Lys74; and (iii) HA-tagged-pGlu25→Thr73. The identification of these three peptide shows that the internal cleavage of the furin propeptide occurs after the -Lys74-Arg75- doublet and that a carboxypeptidase B-like activity in the extract degrades slightly the C-terminus.

Unlike the initial furin propeptide cleavage site (-Arg-Thr-Lys-Arg107-) processed in the RER, the -Lys74-Arg75- doublet is part of a site that lacks a P4 arginine typical of furin substrates, yet possesses a P6 arginine (-Arg70-Gly-Val-Thr-Lys-Arg75-). This kind of cleavage site is also present in a subset of furin substrates preferentially cleaved at acidic pH (Brennan and Nakayama, 1994a,b). To determine the importance of cleavage of the propeptide at Arg75 in the activation of furin, as well as to assess the contribution of Arg70 to this step, site-directed mutagenesis was employed. Replicate plates of BSC-40 cells were infected with vaccinia recombinants expressing either fur/f/haΔtc-k or constructs containing either Arg75→Ala (R_{75}A:fur/f/haΔtc-k) or Arg70→Ala (R_{70}A:fur/f/haΔtc-k) mutations. Crude membrane preparations from each sample were incubated in pH 7.5 or 6.0 buffers, or subjected to limited trypsin proteolysis at pH 7.5 to release the autoinhibitory action of the propeptide (Figure 9). Each construct was proteolytically inactive following incubation at pH 7.5. After incubation at pH 6.0, activation of fur/f/haΔtc-k was observed. By contrast, furin

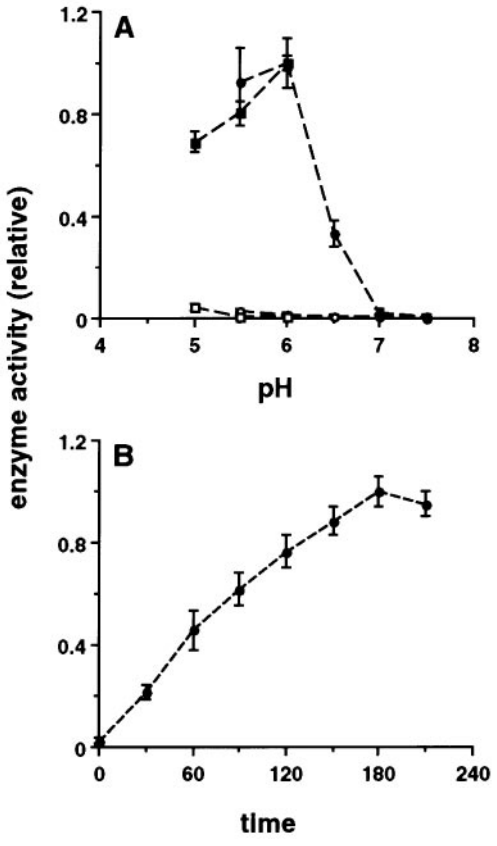

Fig. 4. Effect of pH on furin activation and time-course of activation at pH 6.0. (**A**) Replicate samples of crude membrane preparations from BSC-40 cells infected with VV:hFur/fΔtc-k were resuspendend in 10 mM Bis–Tris buffer (circles) or a 100 mM sodium acetate buffer (squares) at the indicated pH. Samples were pre-incubated at 30°C for 0 min (open symbols) or 150 min (closed symbols) and assayed for proteolytic activity using the Q1 substrate. The data are normalized to the peak activity at pH 6.0. (**B**) Crude membrane preparations of BSC-40 cells infected with VV:hFur/fΔtc-k were resuspendend in a 10 mM Bis–Tris assay buffer, pH 6.0 and pre-incubated at 30°C for up to 210 min. Following pre-incubation the furin activity in each sample was determined using the Q1 substrate. The data were normalized to the peak activity observed at 180 min. Each point in both panels represents the average of three separate determinations of samples assayed in quadruplicate. Bars indicate standard deviations.

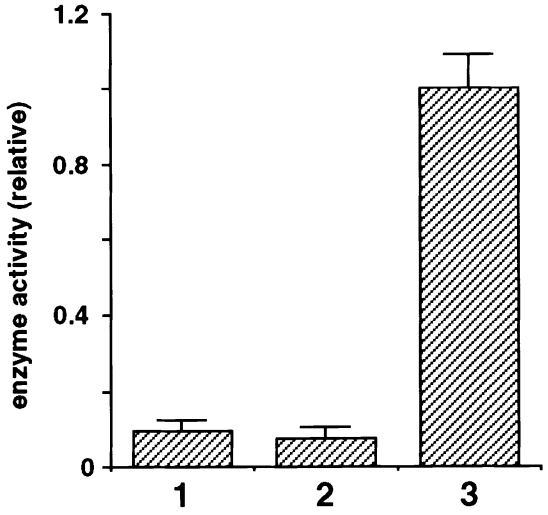

Fig. 5. Limited trypsinization releases inhibition of furin at neutral pH. Crude membrane preparations of BSC-40 cells infected with VV:hFur/fΔtc-k were incubated for 1 h at 30°C at pH 7.5 with no additions (column 1), with 2.5 mM soybean trypsin inhibitor and 0.83 nM bovine trypsin added simultaneously for a mock digestion (column 2), or with 0.83 nM bovine trypsin alone (column 3). Following incubation at 30°C for 1 h, 2.5 mM SBTI was added to the latter sample and all samples were incubated an additional 30 min at 30°C. Following incubation, the furin activity in all samples was determined using the Q1 substrate. Shown are the normalized means from quadruplicate experiments. Bars indicate standard deviations.

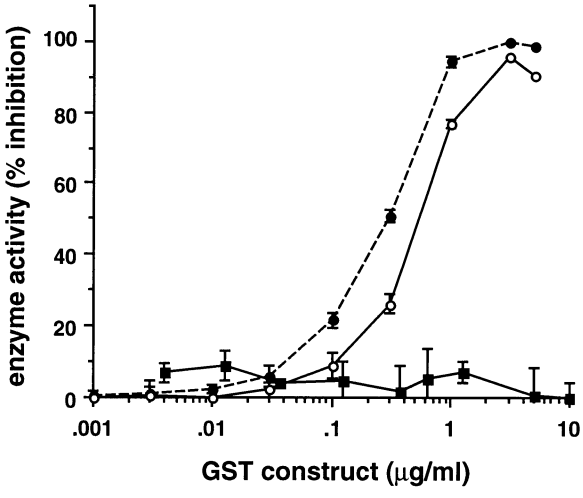

Fig. 6. Inhibition of furin *in trans* by its propeptide. The concentration dependence of furin inhibition by GSTpro (closed circles) or GSTpro/ha (open circles) *in vitro* was determined in 10 mM Bis–Tris assay buffer, pH 7.5 using the fluorescent peptide substrate Boc-Arg-Val-Arg-Arg-4-methyl-coumaryl-7-amide. The $K_{0.5}$ of furin inhibition with GSTpro was 14 nM, and GSTpro/ha was marginally higher. Control samples containing GST alone (closed squares) showed no furin inhibition. Each point represents the average of triplicate samples. Bars indicate standard deviations.

activity was suppressed in samples containing R_{75}A:fur/f/haΔtc-k or, to a slightly lesser extent, R_{70}A:fur/f/haΔtc-k. Following limited trypsinization at neutral pH, however, each of the constructs could be activated. These data show that acid pH-dependent cleavage of the -Lys74-Arg75- doublet is necessary for activation of furin. Furthermore, efficient cleavage at this site requires a P6 arginine (Arg70).

Discussion

Earlier studies revealed that activation of furin requires one or more steps subsequent to the initial cleavage of its N-terminal propeptide (Rehemtulla *et al.*, 1992; Molloy *et al.*, 1994; Vey *et al.*, 1994; Thomas *et al.*, 1995). Here, using an *in vitro* approach, we identify a sequence of events necessary for furin activation. Following translocation of furin into the RER, and concomitant removal of the signal sequence, the proregion is autoproteolytically cleaved at -Arg-Thr-Lys-Arg107$^{\downarrow}$ (Leduc *et al.*, 1992; Molloy *et al.*, 1994; Vey *et al.*, 1994; Creemers *et al.*, 1995). In the neutral environment of the RER, the propeptide remains associated with the endoprotease and functions as an autoinhibitor. Exposure of the furin–propeptide complex to a mildly acidic (pH 6.0) and calcium-containing (low millimolar) environment characteristic of the TGN results in a second cleavage within the propeptide at -Arg70-Gly-Val-Thr-Lys-Arg75$^{\downarrow}$ as determined by mass spectrometry. Mutational analyses showed that both the P1 (Arg75) and P6 (Arg70) residues are essential for the acid pH-dependent

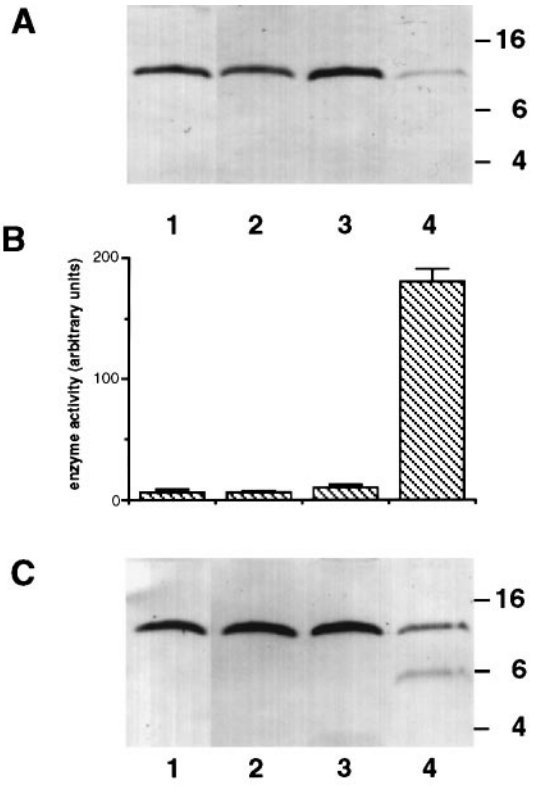

Fig. 7. Fate of the propeptide during furin activation. Equivalent amounts of membrane samples from BSC-40 cells expressing fur/f/haΔtc-k were incubated in 10 mM Bis–Tris assay buffer at pH 7.5 (lanes 1 and 2) or pH 6.0 (lanes 3 and 4) for 0 h (lanes 1 and 3) or 5 h (lanes 2 and 4). Proportionate amounts of the extracts were then divided and analyzed for furin-associated propeptide, enzymatic activity and total propeptide present. (**A**) Proteolytically mature recombinant furin was immunoprecipitated with mAb M1, separated on a 15% SDS–peptide gel, transferred to nitrocellulose, and probed with mAb 12CA5 to identify co-immunoprecipitating HA-tagged propeptide. (**B**) Furin activity in the samples in (A) was determined using the Q1 substrate. (**C**) Extracts from the activation assay were analyzed directly by SDS–peptide gel electrophoresis, transfer to nitrocellulose, and HA-tagged propeptide immunostaining with mAb 12CA5. Molecular weight markers (labeled in kDa) migrated on gels as indicated at the right of (A) and (B). Bars indicate standard deviations.

activation of furin. Following the internal cleavage of the propeptide, the fragments dissociate from furin, permitting the enzyme to cleave substrates *in trans*.

The furin activation pathway determined using the *in vitro* approach reported here is consistent with earlier studies, performed *in vivo*, that described the requirements for activation of this endoprotease (Molloy *et al.*, 1994; Vey *et al.*, 1994). Not only is propeptide cleavage upon entry into the RER a necessary step for activation of the enzyme, it is also a requirement for subsequent export of the membrane-tethered protein (Molloy *et al.*, 1994; Creemers *et al.*, 1995). This requirement suggests that *in vivo* cleavage at Arg107 is necessary for folding of the molecule into a transport-competent form or perhaps facilitates the generation of furin oligomers. None the less, this maturation step, which requires calcium and is sensitive to reducing agents, is alone not sufficient for furin activation. Furthermore, treatment of cells with either brefeldin A or monensin show that furin activation requires exposure to a late Golgi compartment (Vey *et al.*, 1994).

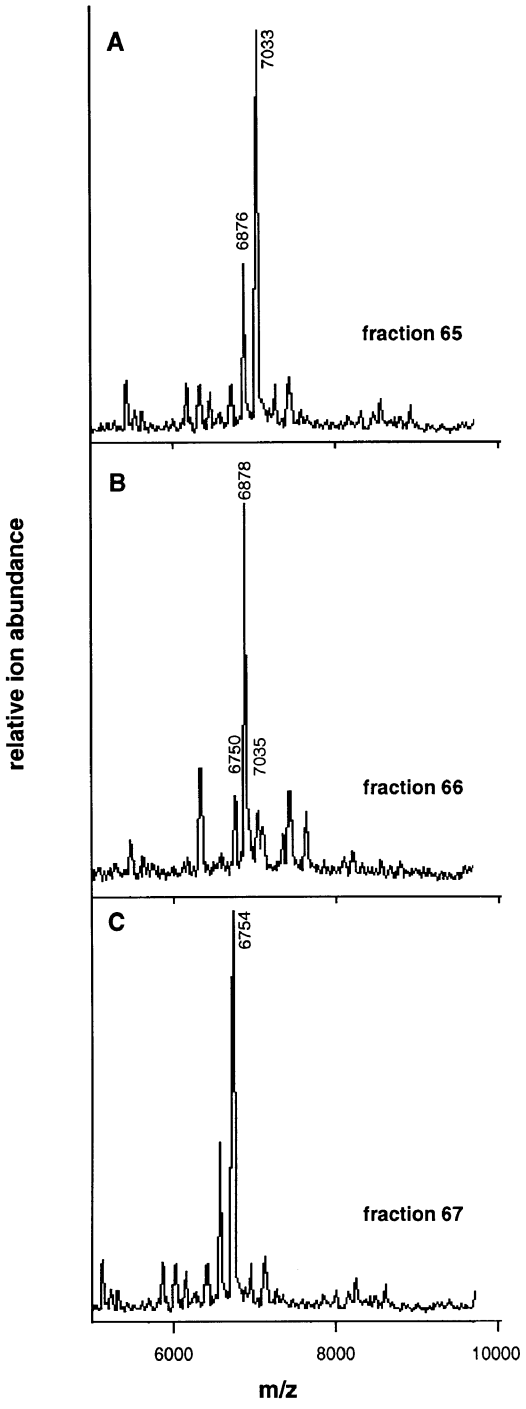

Fig. 8. MALDI–TOF mass spectra of propeptide cleavage products. A crude membrane preparation of BSC-40 cells expressing fur/f/haΔtc-k was incubated in 10 mM Bis–Tris assay buffer, pH 6.0 for 8 h and then fractionated on reversed-phase HPLC. The fractions containing the HA-tagged ~6 kDa band (determined by immunoblotting, data not shown) were subjected to MALDI–TOF MS. In the mass range of this fragment, fraction 65 is seen to contain predominantly the peptide pGlu25→Arg75 (calculated mass = 7036.9), fraction 66 peptide pGlu25→Lys74 (calculated mass = 6880.7), and fraction 67 peptide pGlu25→Thr73 (calculated mass = 6752.5). Electrospray LC/MS of each of these fractions allowed higher precision mass determination as follows: fraction 65 was seen to contain co-eluting species of m/z 1407.9, 1173.7 and 1006.1 (correlating to a mass of 7035.5 a.m.u.); fraction 66 contained co-eluting species of m/z 1377.0, 1147.6, and 983.8 (correlating to a mass of 6880.0 a.m.u.); and fraction 67 contained co-eluting species of m/z 1351.4, 1126.3, and 965.6 (correlating to a mass of 6752.0 a.m.u.).

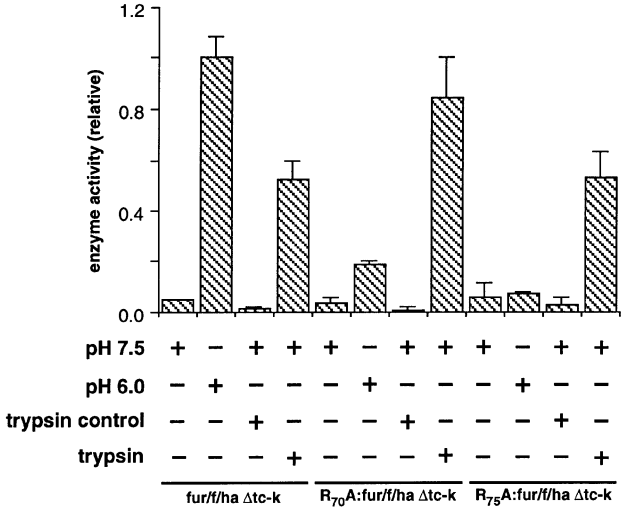

Fig. 9. Activation of R_{70}A:fur/f/haΔtc-k and R_{75}A:fur/f/haΔtc-k *in vitro*. Crude membrane preparations from BSC-40 cells expressing either fur/f/haΔtc-k, R_{70}A:fur/f/haΔtc-k or R_{75}A:fur/f/haΔtc-k were incubated at 30°C in 100 μl Bis–Tris buffer at pH 7.5 or 6.0 with 1 mM 2-mercaptoethanol, 5 mM $CaCl_2$, 500 μM PMSF, 10 μM pepstatin-A, 20 μM E-64 and 50 μg/ml aprotinin for 1.5 h. Alternatively, membrane preparations were incubated with trypsin for 1 h followed by SBTI treatment for 0.5 h or subjected to a mock trypsin digestion (see legend to Figure 6). Following treatment the samples were assayed *in vitro* for furin activity against the Q1 substrate. Each point represents the average of three separate assays. Bars indicate standard deviations.

Our results are consistent in showing the importance of a mildly acid pH to the second site cleavage at -Arg70-Gly-Val-Thr-Lys-Arg75$^{\downarrow}$- and activation of furin. Indeed, by comparing the relative activity of furin constructs containing deletions of the enzyme's cytoplasmic tail, full activation of furin was found to depend on the correct localization and routing within the TGN/endosomal system (Molloy *et al.*, 1994). Whether the need for the correct localization of furin to the TGN is important specifically for the time-dependent internal cleavage, conformational changes in the endoprotease and/or the propeptide, or secondary modification of the protein is not known. However, we have observed that truncated forms of furin are less efficiently sialylated, implying that dwell time in the TGN (including possibly transit through communicating endosomal compartments) affects the maturation of furin (S.S.Molloy and G.Thomas, unpublished results).

The ordered, pH-dependent cleavages of the furin propeptide suggest a mechanism for the compartment-specific autoproteolytic activation of furin *in vivo* which is based on furin's cleavage site specificity. The autoproteolytic and RER-specific initial cleavage occurs at the consensus furin site -Arg104-Thr-Lys-Arg107$^{\downarrow}$- (Leduc *et al.*, 1992). Typical of such sites (-Arg-X-Lys/Arg-Arg-) that are cleaved at either neutral or acidic pH (Hatsuzawa *et al.*, 1992a,b; Molloy *et al.*, 1992), both the P1 (Arg107) and P4 (Arg104) arginine residues are required (Leduc *et al.*, 1992). By contrast, furin is able to cleave some substrates containing the motif -Arg-X-X-X-Lys/Arg-Arg$^{\downarrow}$- only in an acidic environment. For example, proalbumin is cleaved most efficiently at pH 5.5–6.0 at a site requiring both P1 and P6 arginine residues (Brennan and Nakayama, 1994a,b). Here, we show that the measured pH optimum for furin activation *in vitro* (pH 6.0; Figure 4), by virtue of cleavage of the propeptide at -Arg70-Gly-Val-Thr-Lys-Arg75$^{\downarrow}$-, is in close agreement with the pH of the TGN (pH ~6.2; Seksek *et al.*, 1995). Furthermore, mutation of either the P1 (Arg75) or P6 (Arg70) residues to Ala blocks furin activation by acidification but not by trypsinization (Figure 9), demonstrating that cleavage of the propeptide has been blocked, but folding of the enzyme has not been affected. Taken together, these data suggest that furin autoproteolytically cleaves its propeptide at Arg75 within the acidic environment of the TGN, either *in trans* by an associating active furin protein, or by the cognate furin molecule within the propeptide–enzyme bimolecular complex. Whether another protease(s) may be required, as is the case with carboxypeptidase Y activation (Sorensen *et al.*, 1994), is not currently known.

The acidic environment of the TGN may also promote dissociation of the cleaved propeptide fragments from furin. Comparison of the propeptide sequences of the proprotein convertase family shows the presence of multiple conserved histidine residues (reviewed in Siezen *et al.*, 1995, see also legend to Figure 10B). The histidine residues may coordinate a metal atom necessary for enzyme activation or participate in ionic interactions with the mature domain. The pK_a of the ionizable second amino group on the histidine imidazole ring is ~7.0, although this could vary depending on the local microenvironment within the protein (Creighton, 1993). However, within the neutral environment of the RER, these histidine residues should be neutral, while in the acidic environment of the TGN they should become positively charged. This suggests that the acidification necessary to activate furin may contribute to changes in ionic interactions between the propeptide and a metal atom or the propeptide and the enzyme. These changes, together with cleavage of the propeptide at Arg75, could lead to the dissociation of the propeptide fragments from furin, thereby unmasking the protease to act on substrates *in trans*.

In addition to the requirement for acidification, activation of furin may also be regulated by changes in calcium concentration. Whereas maximal activation of furin *in vitro* requires millimolar levels of calcium, propeptide cleavage in the structurally related enzyme PC1/3 *in vivo* requires calcium at only micromolar levels (Vey *et al.*, 1994; J.VanSlyke and G.Thomas, unpublished results). In the RER, the concentration of total lumenal calcium is 3 mM (Sambrook, 1990); however, the free calcium concentration in this compartment is predicted to be ~1 μM (Kendall *et al.*, 1994). By contrast, the free calcium concentration in the TGN is believed to be in the millimolar range (Roos, 1988; Chanat and Huttner, 1991; Chandra *et al.*, 1991; Song and Fricker, 1995). Interestingly, a homology model of furin based on the crystal structures of subtilisin and thermitase has suggested the presence of a high-affinity calcium-binding site (Ca1) and a medium-affinity binding site (Ca2) (Siezen *et al.*, 1994). It is possible that filling of the Ca1 site alone is required for cleavage at Arg107 in the ER, whereas filling of both Ca1 and Ca2 is required for cleavage at Arg75, and hence full activation of furin.

Although activation of the evolutionarily related furin and subtilisin endoproteases shares several properties—including a requirement for autoproteolytic cleavage of propeptides—these processes differ significantly in at least

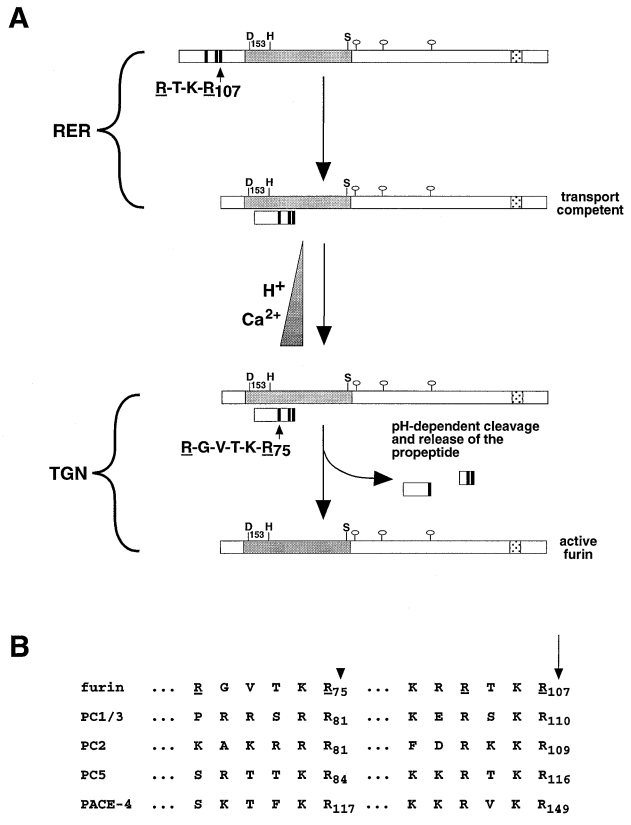

Fig. 10. Model of furin activation *in vivo*. (**A**) The data reported here and in earlier *in vivo* studies (Leduc *et al.*, 1992; Molloy *et al.*, 1994; Vey *et al.*, 1994; Creemers *et al.*, 1995) suggest the following compartment-dependent multi-step model for furin activation. Furin is translocated into the ER and concomitantly the signal sequence is removed at -Ala-Asp-Ala24$^{\downarrow}$-. Following propeptide-mediated folding, furin autoproteolytically cleaves its propeptide at -Arg-Thr-Lys-Arg107$^{\downarrow}$-. In the neutral pH and low free-calcium environment of the RER, the propeptide remains non-covalently associated and functions as an autoinhibitor. Following this initial propeptide cleavage, the furin–propeptide complex is allowed to exit the RER and transit to the TGN. In the mildly acidic and millimolar free-calcium environment of the TGN, the propeptide is cleaved by furin (either the cognate molecule or *in trans* by an associating furin enzyme) at -Arg-Gly-Val-Thr-Lys-Arg75$^{\downarrow}$-. The propeptide fragments generated by this second cleavage bind less tightly (perhaps aided in part by a weakening of histidine-participatory ionic interactions at the acidic pH) and dissociate from the catalytic domain. Hence, furin becomes active to cleave substrates *in trans* (denoted by the highlighting of the catalytic domain) in multiple compartments within the TGN/endosomal system. (**B**) Partial sequence alignment for the propeptides of several proprotein convertases. The numbering used starts with the proposed initiator methionine residues. The spacing between the primary (arrow) and internal (arrowhead) cleavage sites is nearly identical for each of the convertases, ranging in size from 28 to 32 residues. Proximal to the internal cleavage sites are a conserved set of histidine residues present in each endoprotease (corresponding to His66, His69 and His85 in furin; refer to Figure 1) that may participate in either metal-binding or ionic interactions with furin (see Discussion). Arginine residues shown to be essential for the initial and internal cleavages of the furin propeptide are underlined.

three respects. First, furin activation is pH- and thus compartment-dependent, while subtilisin shows no such dependency. The need for this regulation is currently being investigated, but it seems likely that the delay of proteolytic activation prevents the enzyme from cleaving substrates prematurely or inappropriately. Alternatively, the compartment-specific activation of furin may facilitate either the adoption of secondary conformations or oligomerization necessary to the generation of the active species. Second, the fate of the subtilisin propeptide appears to be different from that of furin. The rapid and complete degradation of the subtilisin E propeptide during activation, by an as yet uncharacterized pathway, is likely a result of the broad substrate specificity of this endoprotease. By contrast, we show that dissociation of the furin propeptide is achieved with only limited proteolysis, presumably a result of the restricted cleavage site specificity of this proprotein convertase. Third, the propeptides of proprotein convertases share no sequence similarity with the subtilisin propeptide, despite the homologies among their catalytic domains (Fuller *et al.*, 1989; Seidah *et al.*, 1994; Siezen *et al.*, 1995). This suggests that the roles the furin and subtilisin propeptides play in enzyme maturation may be significantly different. Whether or not the furin propeptide directs folding of the enzyme, as it does with subtilisin, remains to be determined. This role for the propeptide is implied by the finding that truncation of the furin propeptide results in the production of an inactive protease (Rehemtulla *et al.*, 1992).

Although the propeptides of members of the convertase family share no sequence similarity with the propeptide of subtilisin, they are similar to one another (Figure 10B; Siezen *et al.*, 1995). Strikingly, the convertase propeptides are highly charged and contain multiple doublets or clusters of basic amino acids in two distinct regions (Siezen *et al.*, 1995). One basic residue cluster (-Arg-X-Lys-Arg$^{\downarrow}$-) marks the predicted initial sites of autoproteolytic cleavage of the propeptides (Figure 10B). Indeed, biochemical studies show that, as for furin, the propeptides of Kex2p and PC1/3 are cleaved rapidly at this site following deposition of the recently synthesized proenzymes into the RER (Vindrola and Lindberg, 1992; Gluschankof and Fuller, 1994; Goodman and Gorman, 1994; Lindberg, 1994). The second cluster of oligobasic residues present in the propeptides of several other convertases—including those for PC1/3, PC2, PC5/6 and PACE-4—can be aligned with the furin internal cleavage site (Figure 10B). This suggests that the multi-step, pH-dependent activation pathway described here for furin may similarly be used by other members of the family. However, not all proprotein convertases are expected to share all features of the furin activation pathway. For instance, the PC4 and PC7 propeptides lack an internal cluster of basic residues marking the second cleavage site, suggesting that these members of the family may be activated by an alternate pathway. Additionally, although proPC2 undergoes multiple cleavages of its proregion, it also requires the participation of an associating proPC2-binding protein, 7B2 for activation (Braks and Martens, 1994; Matthews *et al.*, 1994). None the less, our results delineating the multi-step activation of furin *in vitro* provide a basis for further work on furin as well as creating a paradigm with which to evaluate the activation pathways of other enzymes in this important converting enzyme family.

Materials and methods

Materials

The protease inhibitors PMSF, pepstatin-A, and soybean trypsin inhibitor (SBTI) were from the Sigma Chemical Company (St Louis, MO). E-64

was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). The peptide substrate Boc-Arg-Val-Arg-Arg-4-methyl-coumaryl-7-amide was from Peptides International (Louisville, KY). The internally quenched substrate Q1, Abz-Arg-Val-Lys-Arg-Gly-Leu-Ala-Tyr(NO_2)-Asp-OH, was a gift from Dr Herbert Angliker (Friedrich Miescher-Institut, Basel). The secreted soluble furin construct Fur713t was purified as described previously (Molloy *et al.*, 1992). Alkaline phosphatase- and horseradish peroxidase-conjugated goat anti-mouse secondary antibodies were obtained from Southern Biotechnology Associates (Birmingham, AL). The mAb 12CA5 directed against the HA epitope was obtained from Boehringer-Mannheim Biochemicals. Purified mAbs M1 and M2 directed against the FLAG epitope were obtained from Kodak-IBI (Rochester, NY).

Cell culture

BSC-40 cells were maintained in minimal essential medium (MEM; Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 25 μg/ml gentamicin as described previously (Thorne *et al.*, 1989; Bresnahan *et al.*, 1990).

Furin constructs and vaccinia virus (VV) expression

The FLAG epitope-tagged furin construct fur/f was generated previously (Molloy *et al.*, 1994). HA-tagged furin constructs were based on the *Eco*RI–*Kpn*I fragment of furin in Bluescript (SK−) as a template, and generated by single-primer (Kunkel) mutagenesis techniques with the oligonucleotide FURIN/HA (5′-CTG CTA GCA GCT GAT GCT CAA GGA TAC CCC TAC GAC GTG CCC GAC TAC GCC CAG GGC CAG AAG GTC TTC-3′). Underlined sequences correspond to native furin sequences between Leu19→Ala24 and Gln25→Phe30. This oligonucleotide introduced the sequence encoding QGYPYDVPDYA (the HA tag is underlined) between the end of the signal sequence and the beginning of the furin propeptide ($A_{24}{\downarrow}Q_{25}G_{26}$; see Results). The additional two residues that are not part of the HA epitope (QG) were introduced to preserve the sequence C-terminal to the signal sequence cleavage site. The full-length furin construct with the HA tag (fur/f/ha) was then generated using the cloning strategy described previously for the construction of fur/f (Molloy *et al.*, 1994). The furin mutants R_{70}A:fur/f/haΔtc-k and R_{75}A:fur/f/haΔtc-k were also generated by single-primer mutagenesis using the primers R70A (5′-CAC TTC TG**G CAT GC**A GGA GTG ACG AAG CGG-3′ and R75A (5′-GTG ACG A**AG GCC T**CC CTG TCG-3′). The nucleotides in each oligo that are complementary to the furin sequence are underlined. The R70A mutation introduced a diagnostic *Sph*I site and the R75A mutation introduced a diagnostic *Stu*I site (shown in bold). The ER-retained furin construct fur/fΔtc-k was generated previously (Molloy *et al.*, 1994) and used to construct R_{70}A:fur/f/haΔtc-k and R_{75}A:fur/f/haΔtc-k by swapping the truncated C-terminus with the KDEL sequence from pZVneo:fur/fΔtc-k in place of the full-length furin tail. All furin constructions were ultimately cloned into the pZVneo vector for generating recombinant vaccinia virus by marker transfer as previously described (VanSlyke *et al.*, 1995).

Glutathione S-transferase (GST) fusion proteins

Sequences encoding the native and HA-tagged furin propeptides were produced by PCR amplification of the appropriate furin construct in pZVneo and inserted between the *Eco*RI and *Bam*HI sites of pGEX 3X (Pharmacia, Piscataway, NJ). GSTpro was constructed using the 5′ primer NNAPROG: 5′-GCG **GGA TCC** AGG GCC AGA AGG TCT TC-3′. GSTpro/ha was constructed using the 5′ primer NHAPROG: 5′-GCG **GGA TCC** TCT ACC CCT ACG ACG TGC CC-3′. The 3′ primer used for the construction of both GSTpro and GSTpro/ha was CPROG: 5′-GCG **GAA TTC** ACC GTT TAG TCC GTC GCT T-3′. The sequences complementary to wild-type or HA-tagged furin are underlined and newly introduced restriction sites are shown in bold. GSTpro and GSTpro/ha were expressed in bacteria and purified according to the manufacturer's instructions (Pharmacia).

Cell fractionation

Crude membrane preparations were made for the analyses of furin constructs by immunoblotting, activation assays, HPLC and mass spectrometry. Confluent BSC-40 cells on 10 cm or 15 cm plates (1 or 2×10^7 cells per plate, respectively) were infected with recombinant vaccinia virus at a multiplicity of infection (m.o.i.) of 5 and incubated for 14–16 h at 37°C in a defined serum-free medium (MCDB202; McKeehan and Ham, 1976). For harvesting, the plates were placed on ice and the medium aspirated. The cells were then removed with a rubber policeman in 2.5 ml (for 10 cm plates) or 5 ml (for 15 cm plates) of 10 mM HEPES, pH 7.2, supplemented with protease inhibitors (0.2 mM PMSF, 1 mM EDTA and 0.01 mM pepstatin-A, and 5 mM E-64). The cells were lysed by repeated passage through a 25-gauge needle followed by centrifugation at 5000 *g* for 5 min at 4°C to remove unbroken cells and nuclei. Membranes were pelleted from 400–500 μl aliquots of the low-speed supernatants by centrifugation at 55 000 r.p.m. in either a TLA 100.1 or TLA 100.3 fixed-angle rotor (Beckman Instruments, Inc., Fullerton, CA) for 1 h at 4°C. Pellets were resuspended in buffers as indicated.

Furin activity assays

All assays were carried out in 100 mM HEPES, pH 7.5, 1 mM 2-mercaptoethanol, 0.5% Triton X-100 and 1 mM $CaCl_2$. Fluorometric assays with 4-methyl-coumaryl-7-amide-containing peptides (AMC substrate) were performed as described previously (Molloy *et al.*, 1992) except when GSTpro or GSTpro/ha were added as indicated. Assays with the internally quenched Q1 substrate were performed essentially as described previously (Angliker *et al.*, 1995) except that a 3 ml reaction volume with 3.8 μM Q1 substrate was used.

Furin activation assays

Membrane pellets were resuspended by trituration into 100 μl of 10 mM Bis–Tris (pH 6.0 or 7.5), 0.5% Triton X-100, 5 mM $CaCl_2$, 1 mM 2-mercaptoethanol. Protease inhibitors were added as indicated. Appropriate substitutions or omissions were made to this buffer to analyze the effect of pH and calcium on the activation of the ER-retained furin constructs. Following resuspension the samples were transferred to 1.5 ml Eppendorf tubes and pre-incubated in a 30°C water bath for the indicated lengths of time. Following incubation, two 30 μl aliquots of the samples were assayed for enzymatic activity against the Q1 substrate.

Immunoprecipitations and immunoblotting

A portion of the membrane pellets resuspended in Bis–Tris buffer was diluted with mRIPA/Ca^{2+} buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM $CaCl_2$). The monoclonal antibody M1 was added (50 μg/ml) and the reaction mixture rotated for 2 h at 4°C. Protein G–Sepharose (Zymed, South San Francisco, CA) was added (30 μl of a 50% slurry) to the immunoprecipitation reactions and incubated 1 h. The Sepharose beads were washed four times with mRIPA/Ca^{2+} buffer before resuspending them in SDS sample loading buffer. Samples were separated on a 15% peptide gel using a SDS–Tris–tricine buffer system (Schägger and von Jagow, 1987; Dayhuff *et al.*, 1992). The proteins were then transferred to nitrocellulose membranes for 30 min at 36 V. The membranes were first blocked in TBST (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% TX-100, 0.01% NaN_3) containing 5% milk and then probed with mAb 12CA5 (0.5 μg/ml final concentration) overnight at 4°C. The secondary antibody (goat anti-mouse–alkaline phosphatase conjugate) was incubated with the blot for 1 h at room temperature and the immunostaining pattern was developed with BCIP and NBT solutions (Zymed). When extracts were to be analyzed directly, portions of the membrane preparation suspensions were mixed with SDS sample loading buffer and subjected to peptide gel electrophoresis and Western blot analysis as described above.

Limited trypsinization of propeptide

Confluent BSC-40 cells were infected with VV:hFur/fΔtc-k and cell membranes were prepared as described above. Membrane pellets were resuspended by trituration in 100 μl of 100 mM HEPES, pH 7.0, 0.5% Triton X-100, 5 mM $CaCl_2$, 1 mM 2-mercaptoethanol. Controls had no subsequent additives, except when 5 μl of 1 mg/ml soybean trypsin inhibitor (SBTI; final concentration = 2.5 μM) along with 2 μl of 1 μg/ml bovine trypsin (for final concentration of 0.83 nM) were added. Trypsin alone was added to the experimental samples and all samples were incubated 1 h at 30°C, after which 5 μl of 1 mg/ml SBTI was added to the experimental samples and all samples were incubated a further 30 min. Enzymatic activity was determined using the Q1 substrate as described above.

Mass spectrometry of propeptide fragment

Forty 15 cm plates of confluent BSC-40 cells (total of 8×10^8 cells) were infected at an m.o.i. of 5 with VV:hFur/f/haΔtc-k construct. At 18 h post-infection, the cells were harvested and a crude membrane preparation made (see above). The membrane preparation was incubated for 8 h at 30°C in 5 ml of 10 mM Bis–Tris, pH 6.0, 5 mM $CaCl_2$, 0.5% Triton X-100, with 0.2 mM PMSF, 0.01 mM pepstatin-A and 5 mM E-64. The clarified supernatant from this incubation was acidified with TFA (final concentration = 0.1%) and run on reversed-phase HPLC

using a Vydac C_4 column, developing the following gradient: 16 to 25% B over 2 min, 25 to 49% B over 75 min, 49 to 90% B over 10 min, where buffer A is 0.1% TFA in H_2O and buffer B is 0.1% TFA in 80% acetonitrile, with a flow rate of 1 ml/min. 1 ml fractions were collected, dried down and resuspended in 100 µl water; 10 µl of each fraction was then run on 15% peptide gel using a SDS–Tris–tricine buffer system, transferred to PVDF membrane and immunoblotted using mAb 12CA5. Fractions containing the ~6 kDa fragment were acidified with acetic acid (final concentration = 10%). 1 µl of each was used for MALDI–TOF analysis on a Voyager Elite (PerSeptive Biosystems, Cambridge, MA) in linear mode with the sample embedded in a sinapinic (3,5-dimethoxy-4-hydroxy cinnamic) acid matrix. Electrospray capillary LC/MS was performed on a Perkin-Elmer/Sciex API-III triple quadrupole with an ionspray source on 25 µl of each sample using R1 resin (PerSeptive Biosystems). At a flow rate of 15 µl/min the chromatography was developed with 0.1% TFA and a gradient of 0 to 60% isopropanol over 60 min, followed by 60 to 100% isopropanol over 5 min. Masses from MALDI–TOF were used to scan the LC/MS data for multiply charged ions of molecular species in the range of interest. The calculation of expected molecular weights was facilitated with the Sherpa data analysis program written by J.Taylor (University of Washington), and all calculations employed average isotope abundance masses.

Acknowledgements

We are grateful to L.Thomas for expert technical assistance throughout all stages of this work. We thank H.Angliker for the Q1 substrate, S.Arch and S.Molloy for critical reading of the manuscript, and members of the Thomas laboratory for helpful discussions. This work was supported by NIH grants DK37274, DK44629 and HD30236 (G.T.). E.D.A. was the recipient of a Tartar Trust Fellowship. J.V. is the recipient of fellowship NRSA DK09069. F.J. is the recipient of an MRC (Canada) fellowship. C.D.T. was the recipient of fellowship NRSA DK09370.

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Received on October 21, 1996; revised on December 6, 1996