The propeptide of furin has multiple roles in guiding the activation of the endoprotease in vivo. The 83-residue N-terminal propeptide is autoproteolytically excised in the endoplasmic reticulum (ER) at the consensus furin site, \(-\text{Arg}^{\text{104}}\)-Thr-Lys-Arg\(^{\text{107}}\) \(-\), but remains bound to furin as a potent autoinhibitor. Furin lacking the propeptide is ER-retained and proteolytically inactive. Co-expression with the propeptide, however, restores trans-Golgi network (TGN) localization and enzyme activity, indicating that the furin propeptide is an intramolecular chaperone. Blocking this step results in localization to the ER-Golgi intermediate compartment (ERGIC)/cis-Golgi network (CGN), suggesting the ER and ERGIC/CGN recognize distinct furin folding intermediates. Following transport to the acidified TGN/endosomal compartments, furin cleaves the bound propeptide at a second, internal P1/P6 Arg site \(-\text{Arg-Gly-Val}^{\text{72}}\)-Thr-Lys-Arg\(^{\text{75}}\) \(-\) resulting in propeptide dissociation and enzyme activation. Cleavage at Arg\(^{\text{75}}\), however, is not required for proper furin trafficking. Kinetic analyses of peptide substrates indicate that the sequential pH-modulated propeptide cleavages result from the differential recognition of these sites by furin. Altering this preference by converting the internal site to a canonical P1/P4 Arg motif \(\text{Val}^{\text{72}} \rightarrow \text{Arg}\) caused ER retention and blocked activation of furin, demonstrating that the structure of the furin propeptide mediates folding of the enzyme and directs its pH-regulated, compartment-specific activation in vivo.

Following correct folding/assembly, many eukaryotic proteins undergo single or multiple endoproteolytic cleavages during transport through the secretory pathway, resulting in the release of smaller, bioactive products. The proprotein convertases (PCs)\(^1\) are a family of calcium-dependent serine endoproteases that catalyze these cleavages at sites containing doublets or clusters of basic amino acids. The PC family is evolutionarily related to bacterial subtilisin and includes seven evolutionary related members expressed in secretory compartments of mammalian cells (see Refs. 1–4 for reviews).

Furin, the most intensively studied member of the PC family, is a type I membrane protein localized primarily to the TGN (5–8). Furin is not statically retained in this compartment, but rather it traffics between two local cycling loops, one at the TGN and the other at the cell surface (9, 10). The dynamic trafficking of furin enables it to cleave and activate numerous cellular and pathogen proproteins in both the biosynthetic and endocytic pathways (reviewed in Refs. 1 and 4). Endoproteolysis of these substrates occurs primarily at the consensus furin cleavage site, \(-\text{Arg-Lys/Arg}-\text{Arg}^{\text{3}},\) containing a P1 and P4 Arg. However, in some cases at acidic pH, furin cleaves substrates at the motif \(-\text{Arg-X-X-Lys/Arg}-\text{Arg}^{\text{2}}\), in which a P6 Arg is present in place of the P4 Arg (11, 12).

Before it can act on proprotein substrates, furin itself must go through a complex process of activation. Furin is translated as an inactive zymogen with an 83-amino acid N-terminal propeptide. Attempts to eliminate or substitute the native furin propeptide region produced inactive enzyme, suggesting that the furin propeptide may play a critical role in folding and activation (13–15). This is consistent with research that shows that the folding of many evolutionarily unrelated classes of protease (e.g. serine-, aspartyl-, cysteinyland metalloproteases) is mediated by (typically N-terminal) propeptides that act as “intramolecular chaperones” (IMCs). IMC-mediated folding has been most thoroughly investigated in the secreted bacterial serine endoproteases 1-lytic protease and subtilisin. These IMCs apparently increase the folding rate of their cognate protease domains by lowering a specific kinetic barrier very late in the folding pathway (reviewed in Refs. 16–20). IMC propeptides are autoproteolytically excised when their cognate enzymes have only partially folded. In subtilisin, propeptide excision initiates significant conformational changes that result in the loss of hydrophobic surface exposure to solvent (21–23). Although no longer covalently attached, these post-excision conformational changes are mediated by the IMC (22).

In the absence of the IMC propeptide, the enzyme folds into an inactive and kinetically stable “molten globule”-like intermediate (16, 25). The addition of the propeptide in trans causes rapid conversion of this folding intermediate to the native state (16, 25, 27, 28). Following excision, IMCs remain noncovalently bound to chromatography; Endo H, endoglycosidase H; ERGIC, ER-Golgi intermediate compartment; CGN, cis-Golgi network; BFA, brefeldin A; CMK, chloromethyl ketone; wt, wild type.
their cognate enzymes, acting as tight binding autoinhibitors (29, 30). Crystal structures reveal that the propeptide C terminus occupies the active site of the cognate proteases, whereas the rest of the propeptide is folded into a domain distant from the active site (31–33). Thus, propeptide-mediated inhibition is a result of the propeptide cleavage site sequence sterically occluding the active site (34). The residues at the excision site play a critical role in mediating IMC action, possibly by guiding the folding of the active site (17, 35). Because IMCs bind and inhibit their cognate proteases, they must be degraded for enzyme activation. This degradation may be autoproteolytic, as has been proposed for subtilisin (31). IMC degradation leaves the cognate protease locked into the native state, which can be metastable (36), by a large kinetic barrier to unfolding (17).

An IMC function for the furin propeptide is consistent with recent work on the maturation of this endoprotease. The furin propeptide is autoproteolytically excised at -Arg-Thr-Lys-Lys

| 107 |

in the ER, and excision is necessary for transport from the early secretory pathway (5, 37–39). However, propeptide excision alone is insufficient for activation, which requires transport to late secretory pathway compartments (5, 38).

Studies in vitro show that after excision the propeptide remains noncovalently bound to furin, acting as a potent auto-inhibitor (K_{i,5} = 14 nM) (13). Exposure of the inactive furin-propeptide complex to conditions characteristic of the microenvironment of the TGN (mildly acidic (pH 6.0) and calcium-containing (low millimolar)) results in a second cleavage within the propeptide at -Arg

| 75 |

Gly-Val-Thr-Lys-Lys

| 75 |

in the ER, and excision is necessary for transport from the early secretory pathway (5, 37–39). However, propeptide excision alone is insufficient for activation, which requires transport to late secretory pathway compartments (5, 38).

Synthesis and Characterization of Internally Quenched Peptides—Peptide synthesis was carried out on an automated peptide synthesizer (Model 431A, Applied Biosystems) using Fmoc solid-phase peptide chemistry until the last step, whereupon protecting groups were used: tert-butyl for Ser, and tert-butyloxyacetyl for Thr. The peptides were cleaved from the resin and deprotected by treating the crude material with a mixture of t-butyloxyacetylamine and t-butyloxyethylamine in a total volume of 100 µl. The reactions were stopped by addition of excess EDTA and the cleavage products were separated by RP-HPLC, and their identities established by mass spectroscopy analysis to determine the site of cleavage by furin, each peptidyl substrate was digested for 15 min at 25°C.

EXPERIMENTAL PROCEDURES

Cell Culture—BSC-40 cells were maintained in minimal essential medium (Invitrogen) containing 10% fetal bovine serum (HyClone) and 25 µg/ml gentamicin as described previously (40). Cell lines were cultured on glass coverslips and grown to 50–80% confluency prior to use. Cells were fixed in 4% paraformaldehyde, and immunofluorescence analysis was performed as described (5).

Experimental Procedures—Furin constructs (31-50) Tyr to the Ala-HMP resin, elongation of the peptide chain was conducted using Fmoc solid-phase peptide chemistry until the last step, whereupon t-butyloxyacetyl-tert-butyloxyethylamine in a total volume of 100 µl. The reactions were stopped by addition of excess EDTA and the cleavage products were separated by RP-HPLC, and their identities established by mass spectroscopy analysis to determine the site of cleavage by furin, each peptidyl substrate was digested for 15 min at 25°C.
FIG. 1. Furin constructs. Fur/f/ha, fur/fpro, fur/fD153N, fur/f/ha, V72R:fur/ f/ha, and R75A:fur/f/ha all have the FLAG epitope tag (diagonal bars) inserted directly after the propeptide cleavage site, such that the N terminus of the FLAG sequence is exposed upon excision. mAb M2 recognizes either the blocked or exposed forms of the FLAG epitope, whereas mAb M1 requires the FLAG epitope at the free N terminus. In fur/f/ha, pro/ha, V72R:fur/f/ha and R75A:fur/f/ha the HA epitope tag (vertical bars) was inserted directly after the signal sequence (black). The HA epitope is recognized by the mAbs 12CA5 and HA.11. The furin catalytic domain is recognized by the antisera PA1–062. The subtilisin-like catalytic domain (hatch marks) and the transmembrane domain (stippled) are indicated. The catalytic triad residues (Asp, His, Ser) are indicated. "Lollipops" denote glycosylation sites. Thick vertical bars indicate propeptide cleavage sites. The propeptide excision and internal cleavage motifs are boxed.

RESULTS

Furin Propeptide Has the Properties of an IMC—To test the possibility that the furin propeptide functions as an IMC, we first examined the requirement of this domain for enzyme activation in vivo by analysis of an epitope-tagged human furin molecule, fur/fΔpro (Fig. 1). This furin construct contains an internal deletion of the entire proregion by fusing the furin signal peptide directly to the FLAG-tagged catalytic domain. For comparison, we generated fur/f/ha, a full-length furin construct containing an HA tag within the proregion and a FLAG tag within the catalytic domain (C-terminal to the propeptide excision site). This double epitope tag strategy permitted simultaneous detection of the furin propeptide and mature domains. Neither epitope tag had detectable effects on the transport and activation of the enzyme (5, 13). Cells expressing either fur/fΔpro or fur/f/ha were analyzed for correct signal/propeptide removal from each construct as determined by Western blot using FLAG-specific mAbs (Fig. 2A). The mAb M2 cross-reacts with all FLAG-tagged furin molecules, whereas mAb M1 requires the tag at the free N terminus (i.e. cleavage of the signal peptide in fur/fΔpro and the propeptide in fur/f/ha, see Fig. 1). The mAb M1 cross-reactivity of the fur/fΔpro and fur/f/ha molecules confirms that the mature proteins possess the same primary amino acid sequences following signal sequence and propeptide (fur/f/ha only) excision (Fig. 2A). Quantitative enzyme assays in vitro revealed the importance of the propeptide to furin activity (Fig. 2B). Extracts from cells expressing fur/f/ha displayed robust activity, whereas fur/fΔpro showed no activity above control samples.

The importance of the propeptide for correct furin localization was also revealed by analyses of the immunofluorescence and glycosylation state. Immunofluorescence analysis showed a distinct localization of fur/f/ha and fur/fΔpro in BSC-40 cells (Fig. 2C). Consistent with the previously demonstrated TGN localization of furin (5), fur/f/ha showed a paranuclear staining pattern with both mature domain (mAb M1) and proregion-directed (mAb HA.11) antibodies. By contrast, fur/fΔpro showed an ER-like staining with mAb M1 including the nuclear envelope and a dispersed reticular pattern that overlapped with the signal sequence receptor, an ER marker. Similar results were obtained with a pulse-chase analysis (Fig. 2D). Following a 30-min pulse with 35S-labeled amino acids, the newly synthesized zymogen and mature forms of fur/f/ha were both sensitive to digestion with endoglycosidase H (Endo H), indicating localization to the ER. After a 3-h chase, most mature fur/f/ha migrates as a larger molecular weight, Endo H-resistant band demonstrating transport to late secretory pathway compartments. By contrast, fur/fΔpro remained in a lower molecular weight, fully Endo H-sensitive form, suggesting that the furin propeptide is required for export of the enzyme from the ER. The relatively high recovery of fur/fΔpro during the entire chase period further indicated that its transport was blocked in the early secretory pathway, because normally a significant portion of furin is proteolytically shed upon delivery to the TGN and is secreted from the cell (reduced signal in fur/f/ha chase lanes).

If, as indicated by these results, the furin propeptide functions as an IMC, then co-expression of the propeptide in trans with fur/fΔpro should rescue fur/fΔpro from the ER and restore enzymatic activity. To test this possibility, a truncated molecule was generated containing only the furin signal sequence followed by the HA epitope-tagged propeptide (pro/ha; see Fig. 1). Cells expressing fur/fΔpro alone or together with pro/ha were incubated with mAb M1 in culture to identify furin molecules recycling from the cell surface. Following fixation, the remaining fur/fΔpro was labeled with mAb M2. Although fur/fΔpro expressed alone or with a control vector did not cycle to the cell surface (as expected based upon its ER localization), co-expression with pro/ha resulted in significant uptake of mAb M1, which gave a punctate, paranuclear stain consistent with TGN localization (Fig. 3A). Thus, pro/ha was able to restore both TGN localization and cell surface cycling of fur/fΔpro. The rescue was not complete because post-fix staining with mAb M2 revealed that some fur/fΔpro remained in the ER (see "Discussion").

To determine whether pro/ha could also rescue fur/fΔpro activity, we used an in vivo pro-β-NGF processing assay. This highly sensitive in vivo assay was used as opposed to the comparatively less sensitive in vitro peptide substrate assay,2 as the rescue of fur/fΔpro localization to the TGN by pro/ha was incomplete. Parallel plates of cells were co-infected with vaccinia recombinants expressing pro-β-NGF and fur/fΔpro alone or with pro/ha. The cells were incubated with [35S]Met/Cys, and

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2 E. D. Anderson and G. Thomas, unpublished results.
secreted pro-β-NGF products were immunoprecipitated and used to quantify processing efficiency (Fig. 3B). Expression of pro-β-NGF with either pro/ha or fur/Δpro separately failed to enhance processing above control levels. By contrast, co-expression of pro/ha with fur/Δpro resulted in significant pro-β-NGF processing. Thus, the furin propeptide expressed in trans can restore both fur/Δpro activity and trafficking.

The IMC-like properties of the furin propeptide suggested that failure of full-length furin to undergo propeptide excision could result in the accumulation of a furin folding intermediate. To test this prediction, we examined the expression of fur/Δ153N, a FLAG-tagged furin construct with an inactivating mutation in the catalytic triad (Asp153→Asn; see Fig. 1). This strategy was based on the finding that active site mutation of the structurally related bacterial subtilisin blocks propeptide excision but not enzyme folding (23, 45). Using Western blotting (Fig. 4A), in vitro activity assays (Fig. 4B), and pulse-chase analysis (Fig. 4C), we verified that fur/Δ153N fails to undergo propeptide excision and thus cross-react with mAb M1. Similar to fur/Δpro, the propeptide-containing fur/Δ153N is inactive and remains fully Endo H-sensitive, consistent with an early secretory pathway transport block. In contrast to fur/Δpro, however, fur/Δ153N displayed a more dispersed punctate staining pattern with a paranuclear component that showed a limited overlap with ERGIC-53, a marker for the ER-Golgi intermediate compartment (ERGIC, Fig. 4D). This limited overlap between fur/Δ153N and ERGIC-53 was confirmed by confocal microscopy (data not shown). The significant
Autoproteolytic Activation of Furin

12883

Autoproteolytic Cleavage of the Internal Furin Propeptide Site at Arg75—Once an IMC has served its function in enzyme folding, this autoinhibitory domain must be removed to permit protease activation. In the case of the degradative bacterial subtilisins, this is achieved via indiscriminate and rapid hydrolysis of the propeptide (reviewed in Ref. 19). By contrast, furin remains inactive following autoproteolytic propeptide excision in the ER (-Arg-Thr-Lys-Arg107 \-), due to stable association with the autoinhibitory propeptide (\(K_{\text{d}} = 14 \text{nM}\) (13)). In \textit{vitro}, activation of this furin-propeptide complex is concomitant with an internal cleavage of the propeptide at the P1/P6 Arg motif, -Arg-Gly-Val-Thr-Lys-Arg75 \-, followed by the dissociation of the cleaved propeptide from furin. Cleavage at this site requires both a P1 and P6 Arg as well as an acidic pH characteristic of the TGN (optimal cleavage at pH 6.0 (13)). Furin cleaves substrates with intact P1/P6 Arg motifs selectively at acidic pH (see the Introduction), raising the possibility that the internal propeptide cleavage is autoproteolytic. To test this possibility, we performed a series of biochemical and cell biological analyses to determine whether cleavage at Arg75 is indeed autoproteolytic and is required for furin activation \textit{in vivo}.

Initially, the sensitivity of internal propeptide cleavage to furin inhibitors was investigated using an ER-localized furin construct, fur/fΔtc-k (see Fig. 1), that undergoes propeptide excision but remains proteolytically inactive because of the stable association of the inhibitory propeptide at the neutral pH of the ER (13). Detergent-solubilized membrane preparations containing fur/fΔtc-k were made and incubated at pH 6.0 in the absence or presence of two potent furin inhibitors, decanoyl-Arg-Val-Lys-Arg-CH\(_2\)Cl, a \(\text{Km}\) inhibitor of all PCs (41), and the selective furin inhibitor, α\(_6\)-PDX (\(K_{\text{f}}\) for furin = 0.6 nM, Fig. 5A and Ref. 41). Consistent with autoproteolysis, both inhibitors blocked the internal cleavage of the furin propeptide and formation of the \(\sim 6\) kDa HA-tagged, N-terminal Glu25 \(\rightarrow\) Arg75 fragment (13). A dilution analysis was also performed to establish whether internal propeptide cleavage was an inter- or intramolecular process. Replicate samples containing fur/fΔtc-k were sequentially diluted (up to 20-fold) and incubated at pH 6.0 for 3 h, during which time furin becomes maximally active \textit{in vitro} (13). Relative furin activity was then determined, and the number of furin active sites measured by titration analysis (Fig. 5B). The observed regression line does not pass through the null point when approaching 0 nM “activable” furin, indicating an intramolecular activation event. However, the rate of activation increases at higher furin concentrations, suggesting an additional intermolecular component. Although the precise \textit{in vivo} concentration of the low abundance endogenous furin is unknown, these results collectively indicate that the enzyme becomes active via a predominantly intramolecular process even at very high local concentrations.

To test directly the hypothesis that autoproteolytic propeptide cleavage at -Arg-Gly-Val-Thr-Lys-Arg75 \- is based on the pH-dependent cleavage site specificity of furin, a kinetic analysis was performed using internally quenched fluorogenic peptide substrates corresponding to the sites of propeptide excision (PS-1) and internal propeptide cleavage (PS-2; see Table I). Consistent with the processing of P1/P4 Arg-containing furin substrates, the PS-1 peptide had a low \(\mu\text{M}\) \(K_{\text{s}}\) value at both neutral and acidic pH but was cleaved slightly more efficiently at pH 7.5. By contrast, the P1/P6 Arg-containing PS-2 peptide showed a marked preference for cleavage at pH 6.0. A much larger \(K_{\text{m}}\) (23.8 \(\mu\text{M}\)) was observed at neutral pH; however, this value was reduced by 3-fold (6.6 \(\mu\text{M}\)) at pH 6.0. Moreover, unlike PS-1, cleavage of PS-2 was slightly more efficient at pH 6 than at pH 7.5. These data support a model in which com-
partment-specific activation of furin is controlled, in part, by different motifs at the two propeptide cleavage sites (see “Discussion”). In addition, the more inefficient cleavage of PS-2 compared with PS-1 may explain, in part, the slower autopro-
teolysis at Arg75 compared with Arg107 during furin activation (see below).

The Slow Autoproteolysis at Arg75 Is Necessary for in Vivo Furin Activation but Not Trafficking—The kinetics of autoproteolytic cleavage and dissociation of the furin propeptide in vivo was determined by pulse-chase, co-immunoprecipitation analysis. Cells expressing fur/ha were pulse-labeled with [3H]Arg/Leu and harvested either immediately or following a chase period of increasing time. Furin-propeptide complexes were then immunoprecipitated with mAb M1, resolved by SDS-PAGE, and processed for autoradiography. Dissociation of the propeptide from furin was quantified by liquid scintillation counting of the excised gel bands (Fig. 5C). The slow time course of propeptide dissociation is consistent with the slower cleavage of the PS-2 substrate in vitro (Table I). Moreover, the inhibition of this step by BFA further indicates a requirement for transport of the furin propeptide complex to TGN/endosomal compartments for the in vivo autoproteolysis at Arg75.

The importance of cleavage at Arg75 to furin activation and sorting was examined in vivo. Biochemical analyses of the biosynthesis and activation of a furin molecule containing an Arg75→Ala substitution (R75A:fur/ha) showed no effect on the initial ER-localized propeptide excision at Arg107, but this substitution still blocked enzyme activation (Fig. 6, A and B). Despite this block in furin activation, pulse-chase analysis showed the Arg75→Ala mutation did not impair transport to the late secretory pathway compartments (Fig. 6C). Moreover, immunofluorescence analyses showed that fur/ha and R75A: fur/ha displayed very similar staining patterns (Fig. 7, Control). The paranuclear staining of both furin mature domains (mAb M1) co-localized with that of their propeptides (HA.11), consistent with the localization of the native and mutated furin-propeptide complexes to the TGN. In addition, a low level of propeptide staining was also detected in the ER of both fur/ha- and R75A:fur/ha-expressing cells. This ER staining likely represents nascent furin molecules, as it was depleted by a brief cycloheximide chase (data not shown). If, however, the cells were treated for 4 h with cycloheximide prior to fixation.
Autoproteolytic Activation of Furin

**FIG. 5.** Autoproteolytic, intramolecular activation of furin. BSC-40 cells were infected with either VV:wt or VV:fur/f/ho-3tc. At 16–18 h post-infection the cells were harvested, and membrane preparations were resuspended in pH 6.0 or 7.5 activation buffer as indicated (10 mM bis-Tris, pH 6.0/pH 7.5, with 5 mM CaCl2 and 1 mM βME). In A, 200 μM α,β-PDX or 200 μM decanoyl-Arg-Val-Lys-Arg-CMK were added prior to incubation at 30 °C for 3 h as indicated. Following incubation, the samples were analyzed for the propeptide by Western blot using the anti-HA mAb 12CA5. The intact propeptide (arrow) and cleaved ∼6-kDa HA-tagged N-terminal propeptide fragment (arrowhead) are indicated. In B, samples were diluted in pH 6.0 activation buffer on ice and subsequently incubated at 30 °C for 3 h. Following incubation, furin activity was determined against a peptide substrate corresponding to the propeptide cleavage site incorporating a P4 Arg (-Arg-X-Lys/Arg-Arg↓). Antibody uptake studies, however, showed indistinguishable staining patterns of internalized mAb M1 for fur/f/ha and R75A:fur/f/ha demonstrating that inhibition of internal propeptide cleavage had no detectable effect on post-TGN trafficking and recycling (Fig. 7, M1 Uptake).

The Sequential Order of Propeptide Cleavages Is Essential for Furin Activation—The slow activation of furin both in vitro and in vivo is presumably due to an inefficient P1/P6 internal propeptide cleavage at Arg75 (see Table I). Therefore, we investigated whether mutating the internal cleavage site to a consensus furin cleavage motif containing a P4 Arg (-Arg-X-Lys/Arg-Arg↓) could accelerate the rate of furin activation. If internal propeptide cleavage in this mutant were efficient at neutral pH, furin might become active in the ER. To test this hypothesis, we first examined the processing kinetics of a fluorogenic peptide substrate incorporating a P4 Arg (i.e. Val72→Arg) (PS-2:V72R; Table I). The introduction of the P4 Arg resulted in an ∼3-fold drop in K± relative to the native internal cleavage site sequence making it comparable with PS-1. Despite the lower K±, the PS-2:V72R Arg substitution still showed a pH-dependent effect on the K± (5.17 μM at pH 7.5 to 1.84 μM at pH 6.0), similar to that seen with PS-2 (∼3-fold). In addition to the change in K±, the efficiency of PS-2:V72R hydrolysis (kcat/K±) increased ∼30-fold over that observed for PS-2 and ∼10-fold over that observed for PS-1.

These kinetic data suggested that cleavage of the internal propeptide site containing the Val72→Arg mutation should indeed be more efficient than that of the native propeptide site at neutral pH in vivo. Therefore, the effects of the P4 Val72→Arg substitution on the biosynthesis and activation of an epitope-tagged furin construct, V72R:fur/f/ha, were determined. Surprisingly, V72R:fur/f/ha failed to undergo efficient propeptide excision and remained largely a zymogen that displayed no proteolytic activity in vitro against a peptide substrate (Fig. 8, A and B). Furthermore, unlike R75A:fur/f/ha, pulse-chase studies showed V72R:fur/f/ha remained predominantly Endo H-sensitive (Fig. 8C), indicating retention in the early secretory pathway. In agreement with the pulse-chase studies, immunofluorescence analysis showed V72R:fur/f/ha was predominantly ER-localized as it co-localized with the signal sequence receptor (Fig. 8D). Interestingly, a small amount of TGN localized, mAb M1-positive (i.e. peptide excised) V72R:fur/f/ha was visible by immunofluorescence (data not shown).
shown) and Western blotting (Fig. 8A), indicating that a minor pool of V72R:fur/ha did mature normally. Although this pool was not detectable by the in vitro activity assay (Fig. 8B), pro-βNF processing in vivo was slightly enhanced in the presence of V72R:fur/ha (data not shown). This suggested that a fraction of the molecules were indeed active. Clearly, however, the majority of V72R:fur/ha is inactive, suggesting that the P1/P6 Arg structure of the internal propeptide cleavage site not only imparts pH dependence to the furin activation process but also is critical for proper IMC function.

Together, these results indicate that V72R:fur/ha is unable to transit out of the ER, implying misfolding and retention by the ER quality control system. To test this possibility, the Val72→Arg mutation was introduced into fur/haΔc-k to facilitate in vitro activation analyses (Fig. 9). We found that the V72R:fur/haΔc-k construct could not be activated in vitro even by limited trypsinolysis, which bypasses the normal pH dependence of propeptide cleavage. This result strongly supports the idea that V72R:fur/ha is predominantly misfolded in vivo and that the ordered, compartment-specific autoproteolysis of the furin propeptide is necessary for enzyme activation.

**DISCUSSION**

Furin activation is a multistep process that requires an ordered pair of compartment-specific autoproteolytic propeptide cleavages to produce the mature active endoprotease. The furin propeptide has several properties of a bona fide IMC. First, furin molecules lacking the propeptide are retained in the ER and are enzymatically inactive (Fig. 2). Second, co-expression of the furin propeptide in trans rescues both its proteolytic activity and its trafficking to the TGN/endosomal system (Fig. 3). Third, blocking propeptide excision results in an ERGIC/CGN localization, suggesting incomplete folding of furin (Fig. 4). Following the rapid propeptide excision at -Arg-Thr-Lys-Arg-107 and transport of the

**TABLE I**

*Peptidyl substrates*

| PS-1 | Abz-Ala-Lys-Arg-Arg-Thr-Lys-Arg↓-Asp-Val-Tyr(NO2)-Ala | 7.5 | 1.99 | 1.90 | 9.58×10^6 | 0.99 | 0.100 |
| PS-2 | Abz-His-Arg-Gly-Val-Thr-Lys-Arg↓-Ser-Leu-Tyr(NO2)-Ala | 7.5 | 23.75 | 6.37 | 2.68×10^6 | 0.27 | 0.462 |
| PS-2:V72R | Abz-His-Arg-Gly-Arg-Thr-Lys-Arg↓-Ser-Leu-Tyr(NO2)-Ala | 7.5 | 5.17 | 41.05 | 7.94×10^6 | 8.259 | 9.439 |

**FIG. 6.** Expression and activity of R75A:fur/ha. BSC-40 cells infected with VV:wt, VV:fur/ha, or VV:R75A:fur/ha were processed for Western analysis (A), in vitro activity assays (B), and pulse-chase analyses (C) as described in Fig. 2.

**FIG. 7.** Furin propeptide localization and propeptide dissociation. BSC-40 cells grown on glass coverslips were infected with either VV:fur/ha or VV:R75A:fur/ha and incubated at 37°C. Where indicated (CHX treated), cycloheximide (10 μg/ml) was added at 2 h post-infection. At 6 h post-infection, the cells were fixed, permeabilized, and incubated with mAb M1 to detect furin mature domain and with HA.11 to detect propeptide; they were visualized with isotype-specific secondary antibodies. In some samples, mAb M1 was added to the culture medium (30 μg/ml) and incubated for an additional hour to label furin molecules recycling from the cell surface prior to fixation (M1 Uptake).
The furin-propeptide complex to the acidic TGN/endosomal system, the propeptide undergoes a slower ($t_{1/2} = 105$ min) autoproteolytic and predominantly intramolecular cleavage at -Arg-Gly-Val-Thr-Lys-Arg$^{75}$ (Fig. 5). Like the initial ER-localized propeptide excision, the autoproteolytic cleavage of the furin propeptide at Arg$^{75}$ is required for enzyme activation (Fig. 6).

Although propeptide excision is necessary for export from early secretory pathway compartments, blocking internal propeptide cleavage (Arg$^{75}$Ala) has no apparent effect on TGN/cell surface cycling (Fig. 7). The importance of the sequences comprising either the rapidly cleaved P1/P4 Arg propeptide excision site or the slowly cleaved P1/P6 Arg internal propeptide cleavage sites for directing activation within the variable pH environment of the secretory pathway was suggested by the kinetics of cleavage of synthetic peptides (Table I). Analysis of a peptide substrate with the internal site of propeptide cleavage mutated to a consensus P1/P4 Arg furin site (Val$^{72}$Arg) showed a greatly increased sensitivity to furin at neutral pH. Surprisingly, this mutation in vivo resulted in near complete block in activation and ER-localization of furin (Fig. 8).

The lack of enzymatic activity of the V72R:fur/f/ha molecule even after trypsinolysis suggested that this mutation causes misfolding (Fig. 9). Together, these results underscore the importance of an ordered, compartment-specific series of activation steps and provide a rationale for the distinct structures of the excision (Arg$^{107}$) and internal (Arg$^{75}$) cleavage sites (summarized in Fig. 10).

Furin Transport Out of the Early Secretory Pathway—Misfolded or unassembled secretory proteins are retained by the cellular quality control system (reviewed in 48). Although most of these proteins are retained in the ER, some escape the ER and transit to post-ER/pre-Golgi compartments, from which they may be retrieved. Our analysis suggests that the ER and ERGIC/CGN may distinguish furin in different folding states. Apparently misfolded furin constructs (i.e. fur/f/ha and V72R:fur/f/ha) are retained in the ER, perhaps by virtue of chaperone binding. Indeed, preliminary co-immunoprecipitation studies show that fur/f/ha and V72R:fur/f/ha are selectively associated with BiP. Chaperone binding to fur/f/ha may account for the incomplete recovery of activity by co-expressed pro/ha (Fig. 3), as BiP may prevent interaction between fur/f/ha and pro/ha. Alternatively, the lack of an ER retrieval signal in pro/ha may have prevented it from reaching a sufficiently high concentration in the ER to fully rescue furin activity. By contrast, the localization of fur/D153N to the ERGIC/CGN suggests that it is partially folded and therefore allowed to progress past the initial ER quality control machinery but not to be transported to late Golgi compartments. We do not know whether fur/D153N is statically retained or recycles between the ER and...
The active conformation (cleavage (deletion of the proregion (fur/f)) signal sequence removal, the propeptide acts as an IMC to facilitate site mutation causes accumulation in the ERGIC/CGN (Ref. 37 with mutations disrupting their propeptide excision site sequence). Blocking the internal cleavage of the propeptide at Arg 75 results in stabilization of the furin propeptide at Arg75 cleavage site (Arg 75) prevents this site from interfering with stabilizing a high energy transition intermediate and thereby lowering a kinetic barrier very late in the folding pathway (reviewed in Refs. 17, 18, and 20). Hence, the propeptide IMCs of both the mammalian PCs and related bacterial proteases may act as “foldases” in contrast to classical chaperones such as BiP. A similar role for the yeast Kex2p propeptide has also been reported (51).

The TGN/Endosomal System and Furin Activation—A requirement for exposure of the furin-propeptide complex to the acidic TGN/endosomal environment for activation is supported by three findings. First, ER-localized furin-propeptide complexes (i.e. fur/fΔtc-k; see Fig. 1) fail to undergo internal propeptide cleavage (13). This step can be promoted, however, by in vitro exposure to mildly acidic pH with maximal cleavage occurring at pH 6.0, in good agreement with the observed pH of the TGN (53). Second, peptide substrates representing the internal propeptide cleavage site are more efficiently cleaved at pH 6.0 than at neutral pH (Table I). Third, internal cleavage and dissociation can be blocked by treatment with BFA, a compound that inhibits ER to TGN transport (Fig. 5C). Our attempts to inhibit internal propeptide cleavage by treatment of cells with deacidifying agents (e.g. chloroquine and bafilomycin A), however, resulted in pleiotropic effects including secondary modifications of furin,2 The incomplete dissociation of the propeptide from furin during activation was surprising (Fig. 5C). Even at long time points (up to 7 h), propeptide dissociation above ~50% was never observed (data not shown).

This may be the result of some furin molecules misfolding due to over-expression, as suggested by the observation that many furin molecules that have undergone propeptide excision remain Endo H-sensitive, indicative of ER retention (Fig. 3). Alternatively, it is possible that over-expression interferes with furinimerization (54) or an unknown but essential secondary modification. Nonetheless, in vitro and in vivo data point to the pH of the TGN/endosomal system as key to governing propeptide cleavage at Arg75.

Compared with the rapid ER-localized propeptide excision (t1/2 < 10 min), the rate of furin activation in vitro and in vivo is slow (t1/2 = 90 (13) and 105 min (this study), respectively). This difference in efficiency of processing at the first and second propeptide cleavage sites may be explained, at least in part, by the kinetics and pH sensitivity of the representative propeptide substrates (Table I). Characteristic of many -Arg-X-Lys/Arg-Arg↓ consensus furin sites, cleavage of PS-1 (reflecting the site of propeptide excision) is relatively efficient at either neutral or acidic pH (low μM Km, relatively high kcat/Km). By contrast, PS-2 cleavage (reflecting the internal P1/P6 Arg propeptide cleavage site) is slower and pH-sensitive with a 3-fold lower Kcat at pH 6.0 (23.8 versus 6.6 μM). Moreover, the use of a P6 Arg instead of a P4 Arg at the internal propeptide cleavage site (Arg75) prevents this site from interfering with ERGIC/CGN or whether fur/Δ153N and ERGIC-53, a potential transport receptor (49, 50), are physically associated.

The possibility that furin folding/activation intermediates are recognized in a compartment-specific manner may explain the differential localization of furin molecules that are caused by excision site mutations versus active site mutations. Both mutations block propeptide excision; however, furin constructs with mutations disrupting their propeptide excision site sequences remain as ER-detained zymogens, whereas the active site mutation causes accumulation in the ERGIC/CGN (Ref. 37 and Fig. 10). This differential localization may be explained by the importance of the propeptide excision site sequence for IMC action as described for α-lytic protease and subtilisin (29, 35) and suggested for Kex2p (51). Thus, mutation of the furin excision site may block the initial ER folding steps, whereas mutation of the catalytic triad may allow these initial steps to occur but block a subsequent ERGIC/CGN folding event. Indeed, mutation of the catalytic triad of subtilisin has been shown not to result in misfolding (23, 45), suggesting that fur/Δ153N may represent the accumulation of a normally occurring, transientsly folded intermediate. This idea is consistent with the proposed mechanism of IMC-mediated folding based on studies of α-lytic protease and subtilisins (reviewed in Refs. 20 and 52). The propeptides of these bacterial enzymes facilitate folding of their cognate proenzymes catalytically by stabilizing a high energy transition intermediate and thereby lowering a kinetic barrier very late in the folding pathway (reviewed in Refs. 17, 18, and 20). Hence, the propeptide IMCs of both the mammalian PCs and related bacterial proteases may act as “foldases” in contrast to classical chaperones such as BiP. A similar role for the yeast Kex2p propeptide has also been reported (51).

**Fig. 10. Summary of furin activation.** Following translation and signal sequence removal, the propeptide acts as an IMC to facilitate folding of the unstructured catalytic domain (gray-striped circle) into the active conformation (black-striped oval). This process is blocked by deletion of the proregion (fur/fΔpro) or by introduction of a canonical P4/P4 Arg furin consensus sequence at the site of internal proregion cleavage (V72Rfur/f/ha), resulting in accumulation of misfolded furin molecules unable to exit the ER. The mutated internal cleavage site apparently out competes the native Arg107 excision site for binding to the catalytic center. However, the aberrantly bound propeptide fails to correctly fold the profurin molecule (inset). After the initial ER folding events, furin undergoes autoproteolytic intramolecular excision of the propeptide at Arg107. The propeptide, however, remains associated with the mature domain functioning as a potent autoinhibitor in trans during transport to the late secretory pathway. Propeptide excision can be blocked by inactivating furin (fur/fD153N) and results in accumulation of an apparent folding intermediate in the ERGIC/CGN. Following propeptide excision, the inactive furin-propeptide complex transits to late secretory compartments (TGN/endosomes) where the relatively acidic pH promotes autotranscytic, intramolecular cleavage of the propeptide at a second, internal site, Arg75. Arg75 cleavage is followed by a rapid dissociation of the propeptide fragments and disassociation of furin. These final activation steps can be blocked by either preventing transport to late secretory compartments (BFA) or by eliminating the internal furin cleavage site (e.g. Arg75→Ala in R75Afur/f/ha). Blocking the internal cleavage of the propeptide at Arg75 results in stabilization of the furin-propeptide complex and prevents activation of furin without altering its trafficking in the TGN/endosomal system.
the cleavage at the propeptide excision site (Arg107). The exquisitely pH-controlled recognition of these two sequences is not consistent with the proposal by Bhattacharya et al. (55) that a simple low pH-induced unfolding of the furin propeptide is solely responsible for cleavage at Arg75 (55). This conflict was underscored by the results of altering the internal cleavage site presented in this paper. Introduction of a P4 Arg at the internal cleavage site (Table I) resulted in a sharp drop in \( K_c \) coupled with a dramatic increase in \( k_{cat}/K_m \) (30-fold). Indeed, the \( k_{cat}/K_m \) of PS-2:V72R was nearly 10-fold greater than that of the propeptide excision site peptide, PS-1. In addition, when this Val72 → Arg mutation was introduced into furin (V72R: fur/f/ha), it inhibited propeptide excision and resulted in ER retention (Fig. 8).

One explanation for the disrupted activation of V72R:fur/f/ha is based on studies of pl-lytic protease and subtilisin. For both bacterial proteases, the integrity of the propeptide excision site sequence is crucial for IMC activity (29, 35). In addition, the subtilisin propeptide is unstructured, folding only by virtue of interaction with the protease domain (27). Thus, in an unstructured propeptide during early folding times the internal cleavage site of V72R:fur/f/ha may out-compete the excision site for access to the partially folded furin catalytic pocket by virtue of its more favorable kinetic properties (Table I). This inappropriate binding would prevent furin from folding properly, leaving the bulk of the enzyme as a misfolded, ER-retained zymogen (see Fig. 10). In a minority of V72R:fur/f/ha molecules, the propeptide excision site interacts correctly with the active site, resulting in their proper folding (Fig. 8 and data not shown). The scarcity of this species, however, precluded biochemical characterization. Importantly, these data may explain why the propeptide excision site interacts correctly with the active site, while its more favorable kinetic properties (Table I). This inappropriate binding would prevent furin from folding properly, leaving the bulk of the enzyme as a misfolded, ER-retained zymogen.

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Autoproteolytic Activation of Furin

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