Aggregation As a Determinant of Protein Fate in Post-Golgi Compartments: Role of the Luminal Domain of Furin in Lysosomal Targeting

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Abstract. The mammalian endopeptidase furin is a type 1 integral membrane protein that is predominantly localized to the TGN and is degraded in lysosomes with a $t_{1/2}$ = 2–4 h. Whereas the localization of furin to the TGN is largely mediated by sorting signals in the cytosolic tail of the protein, we show here that targeting of furin to lysosomes is a function of the luminal domain of the protein. Inhibition of lysosomal degradation results in the accumulation of high molecular weight aggregates of furin; aggregation is also dependent on the luminal domain of furin. Temperature and pharmacologic manipulations suggest that furin aggregation occurs in the TGN and thus precedes delivery to lysosomes. These findings are consistent with a model in which furin becomes progressively aggregated in the TGN, an event that leads to its transport to lysosomes. Our observations indicate that changes in the aggregation state of luminal domains can be potent determinants of biosynthetic targeting to lysosomes and suggest the possible existence of quality control mechanisms for disposal of aggregated proteins in compartments of the secretory pathway other than the endoplasmic reticulum.

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The endopeptidase furin is a type I integral membrane glycoprotein (see scheme in Fig. 1 *a*), which, at steady state, is predominantly localized to the TGN (Bosshart et al., 1994; Molloy et al., 1994; Schäfer et al., 1995; Shapiro et al., 1997). Furin has also been detected within compartments of the endosomal–lysosomal system (Bosshart et al., 1994; Sariola et al., 1995), which are most likely intermediates in the cycling of the protein between the TGN and the plasma membrane (Chapman and Munro, 1994; Molloy et al., 1994), or in its transport to lysosomes for degradation (Bosshart et al., 1994).

During its transit through the secretory pathway, furin undergoes several posttranslational modifications. In the ER, nascent furin receives three N-linked oligosaccharides chains (see Fig. 1 *a*, *N-CHO*) and undergoes sequential cleavage of its NH_2-terminal signal peptide (Fig. 1 *a*, *SP*) and pro-region (Fig. 1 *a*, *PR*) (Leduc et al., 1992; Rehemtulla et al., 1992; Molloy et al., 1994). The pro-region fragment remains noncovalently bound to furin as the protein moves from the ER to the Golgi complex (Anderson et al., 1997). In the TGN, there is an additional cleavage in the pro-region fragment that causes its dissociation from the rest of the molecule (Anderson et al., 1997). Furin then establishes transient residence within the TGN and engages in cycling between the TGN and the plasma membrane (Chapman and Munro, 1994; Molloy et al., 1994). Somewhere along this cycling pathway, a fraction of the furin molecules are cleaved near the luminal–transmembrane boundary, resulting in the release of the soluble luminal domain into the extracellular space (Wise et al., 1990; Bosshart et al., 1994; Molloy et al., 1994; Vey et al., 1994; Creemers et al., 1996). The population of furin that is not cleaved eventually becomes degraded by a lysosomal process with a $t_{1/2}$ = 2–4 h (Bosshart et al., 1994).

In view of the complexity of furin's trafficking patterns, it is not surprising that the protein contains several post-Golgi sorting determinants. The cytosolic domain of furin has at least two sorting signals that are responsible for its localization to the TGN and its internalization and retrieval from the plasma membrane (Bosshart et al., 1994; Chapman and Munro, 1994; Molloy et al., 1994; Schäfer et al., 1995; Voorhees et al., 1995). One is a tyrosine-based signal (YKGL; residues 758–761) (Schäfer et al., 1995; Voorhees et al., 1995) that is similar to other signals that

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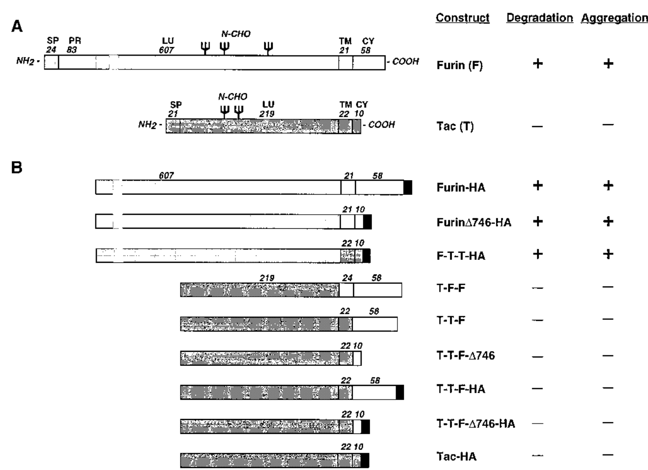

Figure 1. Schematic representation of constructs used in this study and summary of results. (*A*) The diagrams show the position and number of amino acid residues of the signal peptide (*SP*), pro-region (*PR*), luminal (*LU*), transmembrane (*TM*), and cytosolic (*CY*) domains of furin and Tac. The figure also indicates the approximate positions of sites of N-glycan addition (*N-CHO*) in both molecules. The gap in the furin luminal domain represents a segment that was omitted to draw the schemes to scale. (*B*) The diagrams represent the structures of different furin-Tac chimeras after cleavage of the signal peptide and pro-region segments. In naming the chimeras, *F* and *T* were used to designate domains derived from furin and Tac, respectively. Some of the constructs were tagged by addition of an HA epitope (YPYDVPDYA) at the COOH terminus, as shown in the figure. In some constructs, a FLAG epitope (DYKDDDDK) was used in place of the HA epitope. The number of residues in the transmembrane and cytosolic domains of the chimeras are indicated.

interact with the medium chains of the clathrin-associated adaptor complexes AP-1 and AP-2 (Ohno et al., 1995, 1996; Boll et al., 1996; for review see Marks et al., 1997). The other signal is a sequence rich in acidic amino acid residues (WQEECPSDSEEDEGRGER; residues 766–783) (Schäfer et al., 1995; Voorhees et al., 1995) that becomes phosphorylated on serine residues by casein kinase II (Jones et al., 1995; Takahashi et al., 1995). The phosphorylated acidic sequence has recently been shown to interact with the AP-1 adaptor complex (Dittié et al., 1997).

Previous work from our laboratory suggested that the two cytosolic sorting signals are not the only determinants of furin trafficking in post-Golgi compartments. Indeed, deletion of all but 11 amino acids from the cytosolic tail, including the tyrosine-based and acidic signals, resulted in loss of TGN localization; yet, the truncated protein remained mostly intracellular (Bosshart et al., 1994). Like full-length furin, the truncated protein was found to be degraded in lysosomes at a relatively rapid rate (Bosshart et al., 1994). Addition of the same 11 cytosolic amino acids of furin to the luminal and transmembrane domains of the α chain of the interleukin-2 receptor (Tac),[1] a plasma membrane protein, did not alter the cell surface localization and turnover of the protein, suggesting that this sequence did not have lysosomal targeting activity per se (Bosshart et al., 1994). These observations pointed to the existence of additional targeting information within the luminal and/or transmembrane domains of furin. In the present study, we demonstrate that the rapid turnover of furin is a property of the luminal domain of the protein. Furthermore, we show that lysosomal targeting mediated by the furin luminal domain correlates with formation of high molecular weight aggregates, a process that most likely occurs within the TGN. Thus, our results suggest that progressive aggregation of the luminal domain of furin in the TGN results in its delivery to lysosomes for degradation. These observations emphasize the importance of the aggregation state of proteins in post-Golgi trafficking pathways and suggest the possible existence of quality control mechanisms for disposal of aggregated proteins in compartments of the secretory pathway other than the ER (Hammond and Helenius, 1995).

Materials and Methods

Recombinant DNA Procedures

Schematic representations of recombinant constructs used in this study are shown in Fig. 1. Mouse furin (Hatsuzawa et al., 1990) constructs tagged with either the FLAG (Hopp et al., 1988) or hemagglutinin (HA) (Wilson et al., 1984) epitopes and cloned into the pCDL-SRα vector (Takebe et al., 1988) were described previously (Bosshart et al., 1994). The promoter region of pCDL-SRα has SV40 early promoter and human T-cell lymphotrophic virus I long terminal repeat sequences that drive expression of the transgenes in cells from different mammalian species. Tac (Leonard et al., 1984) constructs and Tac-furin chimeras cloned into pCDM8 (Seed, 1987) were also described previously (Bosshart et al., 1994; Voorhees et al., 1995). The pCDM8 plasmid has a human cytomegalovirus promoter that yields good expression levels in primate cells. The construct F-T-T-HA encodes a fusion of residues 1–714 of mouse furin with residues 241–273 of Tac, plus the HA epitope; the construct was made using the double PCR method of Higuchi et al. (1988), and then cloned into pCDL-SRα.

Cells

Rat basophilic leukemia (RBL) cells (clone 2H3) were provided by H. Metzger (National Institutes of Health [NIH], Bethesda, MD). HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). Swei cells (human B-lymphoblastoid) were the gift of M. Marks (University of Pennsylvania, Philadelphia, PA). All cells were cultured in DME (Biofluids, Inc., Rockville, MD) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Stable transfection of RBL cells and transient transfection of HeLa cells were done as described previously (Humphrey et al., 1993; Bosshart et al., 1994; Marks et al., 1995). Single-cell clones of stably transfected RBL cells were isolated by limiting dilution and screened by immunofluorescence microscopy. Only clones that expressed moderate levels of the transfected constructs were used. RBL lines expressing Tac or a Tac-DKQTLL construct (Letourneur and Klausner, 1992) were the gift of R. Klausner (NIH).

Transient Transfections

HeLa cells were transfected using calcium phosphate precipitation as described (Marks et al., 1996). Typically, 10–40% of the cells were transfected using this procedure. Cells were used 36–40 h after transfection for metabolic labeling or immunofluorescence microscopy.

Antibodies

The antibodies Fur1, Fur2 (Bosshart et al., 1994), and DC16 (a gift from R. Angeletti, Albert Einstein College of Medicine, New York) directed to

1. *Abbreviations used in this paper*: DTSSP, dithio bis-sulfosuccinimidylpropionate; HA, hemagglutinin epitope; MHC, major histocompatibility complex; RBL, rat basophilic leukemia; Tac, α chain of the interleukin-2 receptor.

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different luminal epitopes of furin, were described before (Bosshart et al., 1994, Shapiro et al., 1997). The antibody M2 to the FLAG epitope was obtained from International Biotechnologies, Inc. (New Haven, CT), and the antibody HA-11 to the HA epitope was obtained from BAbCO (Richmond, CA). The mouse monoclonal antibody 7G7.B6 (referred to as 7G7; Rubin et al., 1985), directed to a luminal epitope of the human Tac antigen, was produced from a hybridoma clone obtained from the American Type Culture Collection. A polyclonal antibody to lamp-1 was a gift from M. Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA).

Immunofluorescence Microscopy

Cells were grown to 70–80% confluence on glass coverslips and fixed for 15 min at room temperature with 2% formaldehyde in PBS. After washing in PBS, the cells were incubated for 1 h with primary antibodies in 0.1% saponin/PBS. After rinsing in PBS, cells were further incubated for 30 min with a mixture of tetramethyl rhodamine– and fluorescein-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) in 0.1% saponin/PBS. The coverslips were washed once more in PBS and mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates Inc., Birmingham, AL). Samples were examined with a confocal microscope (Zeiss LSM 410, Carl Zeiss Inc., Thornwood, NY).

Metabolic Labeling, Immunoprecipitation, and Electrophoresis

Cells were pulse labeled for 30 min with [^{35}S]methionine (EXPRE^{35}S^{35}S; DuPont-NEN, Boston, MA) in methionine-free medium, chased for various times in complete medium, and proteins were immunoprecipitated with antifurin or anti-Tac antibodies, as described in previous publications (Bosshart et al., 1994; Voorhees et al., 1995). Furin constructs were immunoprecipitated with mixtures of antibodies, including Fur1, Fur2, DC16, and, whenever appropriate, anti-FLAG (M2) or anti-HA (HA-11) antibodies. Immunoprecipitates were separated by SDS-PAGE on either 8% acrylamide or 4–20% acrylamide gradient gels and labeled bands revealed by fluorography.

Sedimentation Velocity Analyses

Metabolically labeled cells were solubilized for 15 min at 4°C in 1% (wt/vol) Triton X-100, 0.5% sodium deoxycholate, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4 (lysis buffer). Lysates (0.5 ml) were cleared by centrifugation for 15 min at 15,000 *g* at 4°C, applied to the top of 12-ml, linear 5–20% (wt/vol) sucrose gradients, centrifuged for 16 h at 4°C in a SW41 rotor at 39,000 RPM (188,000 *g*), and fractions collected as described (Bonnerot et al., 1994). Each of 15 fractions collected from the gradients was subjected to immunoprecipitation with antibodies to furin, Tac, or epitope tags. Peak fractions for migration of a human major histocompatibility complex (MHC)-class I molecule (~57 kD) and the human transferrin receptor (~190 kD), used as size markers for integral membrane proteins, were identified by immunoprecipitation of gradient fractions from metabolically labeled Swei cells, as described (Bonnerot et al., 1994).

Chemical Cross-linking

Metabolically labeled cells were solubilized as described above, except that Hepes-NaOH, pH 7.4, was used in place of Tris-HCl in the lysis buffer, because the amino groups in Tris react with the cross-linking reagent. The cleared cell lysate (0.5 ml) was incubated at room temperature with different concentrations (0.1–3 mM) of dithio bis-sulfosuccinimidyl-propionate (DTSSP). After 20 min, the reactions were quenched with an equal volume (0.5 ml) of Tris-containing lysis buffer, and then the cross-linked samples were subjected to immunoprecipitation. DTSSP has an intermolecular disulfide bond between its reactive groups that allows the cross-link between the proteins to be broken by treatment with reducing agents. Thus, all immunoprecipitates were run on SDS-PAGE under non-reducing conditions to evaluate the extent of cross-linking, and under reducing conditions to demonstrate that the cross-linked protein corresponded to furin.

Pharmacologic Manipulations

Lysosomal protein degradation was inhibited by incubation with either 50 mM NH_4Cl, 200 μM chloroquine or LPEM mixture (100 μg/ml leupeptin, 100 μg/ml pepstatin, 100 μg/ml E64, and 20 mM methionine methyl ester) (Bosshart et al., 1994) in complete medium containing 20 mM Hepes-NaOH buffer, pH 7.1. Aluminum fluoride (AlF_4^-) was generated by the addition of 1 μl/ml 30 mM to $AlCl_3$ to complete medium containing 20 mM Hepes-NaOH buffer, pH 7.1. After vortexing, 10 μl/ml of 1 M NaF was added to the medium, vortexed, and then immediately added to cells.

Results

Lysosomal Degradation of Furin

As previously demonstrated (Bosshart et al., 1994), a furin-FLAG construct expressed stably in RBL cells was degraded with a $t_{1/2}$ = 2–4 h (Figs. 2 and 3). A small amount of an ~80-kD cleavage product of furin, observed after long exposure of autoradiograms, was released into the medium; however, this species did not account for the rapid loss of furin from the cells. The degradation of furin was not caused by the presence of the FLAG epitope, as furin-HA in RBL cells (Fig. 4) and untagged furin in HeLa cells (Fig. 2) were degraded with similarly rapid rates. Degradation occurred after transport through the Golgi complex (Bosshart et al., 1994), and was inhibited by NH_4Cl (Figs. 2 and 4) and other inhibitors of lysosomal proteolysis (Bosshart et al., 1994), suggesting that it took place within lysosomes. Thus, the relatively rapid turnover of furin appears to be an intrinsic property of the protein, independent of the cells in which it is expressed and of the presence or absence of epitope tags.

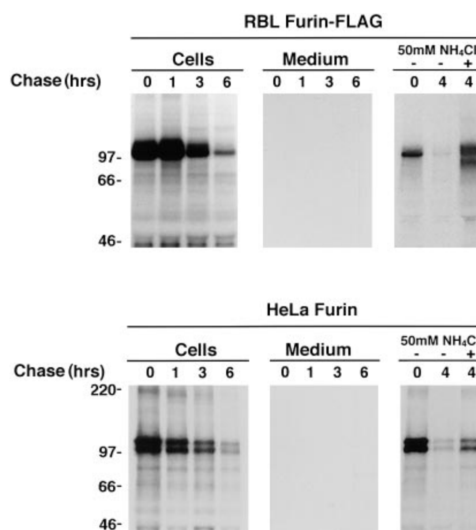

Figure 2. Lysosomal turnover of furin in transfected cells. Stably transfected RBL cells expressing furin-FLAG and transiently transfected HeLa cells expressing untagged furin were analyzed by pulse chase metabolic labeling as described in Materials and Methods. Cells were pulse labeled for 30 min with [^{35}S]methionine and chased for various times in the absence (−) or presence (+) of 50 mM NH_4Cl as indicated in the figure. Furin-FLAG was isolated from cells and media by immunoprecipitation with a mixture of antibodies, including Fur1, Fur2, DC16, and M2; untagged furin was isolated using the same antibody mixture, except for M2. The presence of an ~80-kD soluble furin species in the media was detected upon prolonged exposure of the autoradiograms.

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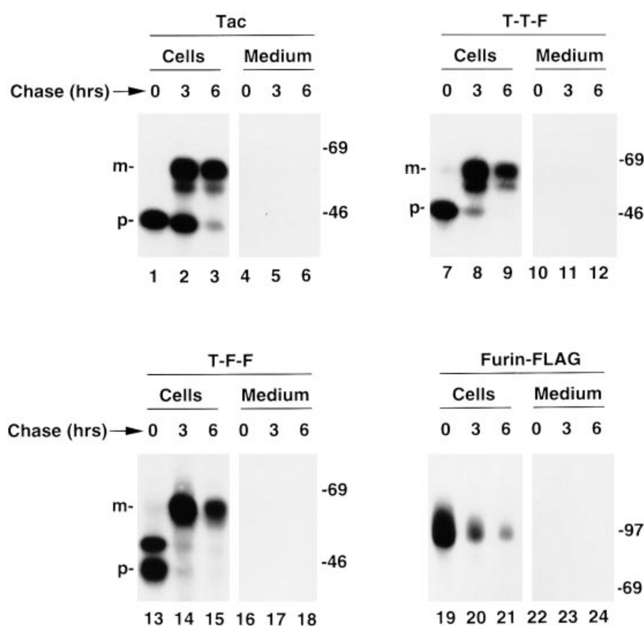

Figure 3. Rapid turnover of furin is not mediated by the transmembrane and cytosolic domains. Stably transfected RBL cells expressing Tac, T-T-F, T-F-F, or furin-FLAG (see Fig. 1 for structures) were pulse labeled with [^{35}S]methionine for 30 min and chased for 0, 3, or 6 h. Tac species were isolated by immunoprecipitation with the monoclonal antibody 7G7 and furin species with a mixture of Fur1, Fur2, and DC16. The symbols *p* and *m* point to the ER precursor and Golgi-processed, mature forms of the Tac proteins, respectively. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) are shown at right.

Rapid Degradation of Furin Is Not Mediated by Its Cytosolic or Transmembrane Domains

To map the topologic domain(s) of furin that determine its rapid lysosomal turnover, we examined the fate of chimeric proteins made by exchanging domains between furin and the long-lived plasma membrane protein, Tac (Fig. 1 *a*). One of the chimeras, T-T-F, consisted of the luminal and transmembrane domains of Tac and the cytosolic domain of furin; another chimera, T-F-F, had the luminal domain of Tac and the transmembrane and cytosolic domains of furin (Fig. 1 *b*). Pulse-chase analyses showed that both chimeras were much more stable than furin-FLAG (Fig. 3). Therefore, the cytosolic and transmembrane domains of furin could not target a reporter protein to lysosomes and were thus not likely responsible for the rapid turnover of furin.

The Luminal Domain Is Largely Responsible for the Rapid Lysosomal Degradation of Furin

Further investigation of the domain(s) of furin that mediate its rapid turnover were performed with chimeric proteins containing the furin luminal domain (the structures are shown in Fig. 1 *b*), expressed in stably transfected RBL cells. As previously shown (Bosshart et al., 1994), furin-HA was rapidly degraded (Fig. 4). Deletion of residues 747–793 from the cytosolic domain of furin, which include both the tyrosine-based and acidic signals (Schäfer et al., 1995; Voorhees et al., 1995), did not prevent degradation (Fig. 4, *furinΔ746-HA*). Moreover, a chimera consisting of the luminal domain of furin, the transmembrane and cytoplasmic domains of Tac and the HA epitope (*F-T-T-HA*) was also rapidly degraded (Fig. 4). The degradation of these proteins was not because of the presence of the HA epitope, since placement of this epitope at the COOH terminus of analogous constructs having the luminal domain of Tac produced proteins that were stable over the time span of the experiments (Fig. 4, *T-T-F-HA*, *T-T-FΔ746-HA,* and *Tac-HA*). Thus, the only structural difference between F-T-T-HA (which is rapidly degraded) and Tac-HA (which is stable) is the in-luminal domain of the proteins. The ability of NH_4Cl (Fig. 4, lanes 13–72) and other inhibitors of lysosomal proteolysis (not shown) to inhibit degradation of proteins having the furin luminal domain, confirmed that the degradation occurred by a lysosomal pathway. Taken together, the experiments shown in Figs. 3 and 4 indicate that the luminal domain of furin is both necessary and sufficient for targeting degradation.

Morphologic Evidence That the Luminal Domain of Furin Targets It to Lysosomes

The transport of furin to lysosomes and the role of the luminal domain in this process were also examined by immunofluorescence microscopy. As previously reported (Bosshart et al., 1994), furin-HA was localized to a juxtanuclear structure characteristic of the TGN (Fig. 5 *A*). After a 3-h incubation with NH_4Cl however, a large fraction of furin-HA was localized to lysosomes (Fig. 5 *B*), as demonstrated by costaining with an antibody to the lysosomal membrane protein lamp-1 (Fig. 5 *C*). F-T-T-HA displayed weak staining of the ER and the Golgi complex in untreated cells (Fig. 5 *D*). Treatment with NH_4Cl resulted in an increase of the fluorescent signal and the appearance of the protein in lamp-1–positive vesicles (Fig. 5, *E* and *F*). As a control for the influence of the HA epitope on protein localization, we examined the distribution of Tac-HA. This protein was found at the plasma membrane in both untreated (Fig. 5 *G*) and NH_4Cl-treated cells (Fig. 5 *H*), confirming that the HA epitope does not mediate lysosomal targeting. Since F-T-T-HA and Tac-HA differ only in the luminal domains, we concluded that the accumulation of F-T-T-HA in lysosomes is a function of the luminal domain of furin. These morphological observations are consistent with the biochemical data showing that a significant fraction of newly synthesized furin is transported to lysosomes and that the lysosomal targeting is largely mediated by the luminal domain of the protein.

Inhibition of Lysosomal Degradation Reveals the Formation of High Molecular Weight Aggregates of Furin

The finding that the determinant of rapid turnover of furin is contained within the luminal domain was unexpected because most lysosomal targeting determinants identified to date map to cytosolic domains (Mellman, 1996; Marks et al., 1997). A possible explanation for our observations was that the luminal domain of furin harbored a specific sorting signal for targeting to lysosomes. Alternatively, delivery of furin to lysosomes could be mediated by a change in the physical-chemical properties of the protein at the

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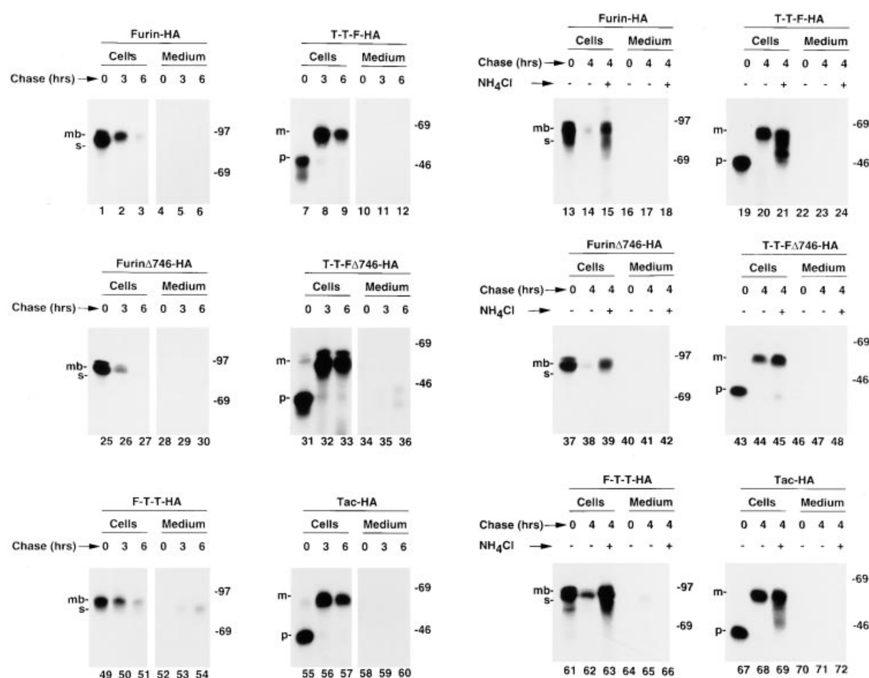

Figure 4. The luminal domain of furin is necessary and sufficient for its rapid lysosomal degradation. Stably transfected RBL cells expressing various furin and Tac chimeras (see Fig. 1 for structures) were pulse labeled with [^{35}S]methionine for 30 min and chased for different times in the absence (−) or presence (+) of 50 mM NH_4Cl. Tac and furin species were isolated with the same antibodies mentioned in the legend to Fig. 3. The positions of relative molecular mass markers (expressed as $10^{-3} \times M_r$) are shown at right. The symbols *p* and *m* point to the ER precursor and Golgi-processed, mature forms of the Tac proteins, respectively. The symbols *mb* and *s* mark the positions of membrane-bound (~100 kD) and soluble (~80 kD) forms of furin, respectively, as previously described (Bosshart et al., 1994).

TGN or at some post-TGN compartment. We decided to examine the aggregation state of furin, because formation of high molecular weight aggregates has been proposed to contribute to protein retention within the ER lumen (for review see Rose and Doms, 1988; Hurtley and Helenius, 1989) and to precede the nonlysosomal degradation of various proteins (Delahunty et al., 1993; Bonnerot et al., 1994). Furthermore, aggregation has been implicated in diverting proteins from the TGN to the regulated secretory pathway (for review see Bauerfeind and Huttner, 1993).

The aggregation state of various furin and Tac constructs was analyzed by sedimentation velocity on sucrose gradients (Martin and Ames, 1961). Newly synthesized furin-FLAG sedimented with a size compatible with it being a small oligomer (i.e., a dimer or a trimer [The type of gra-

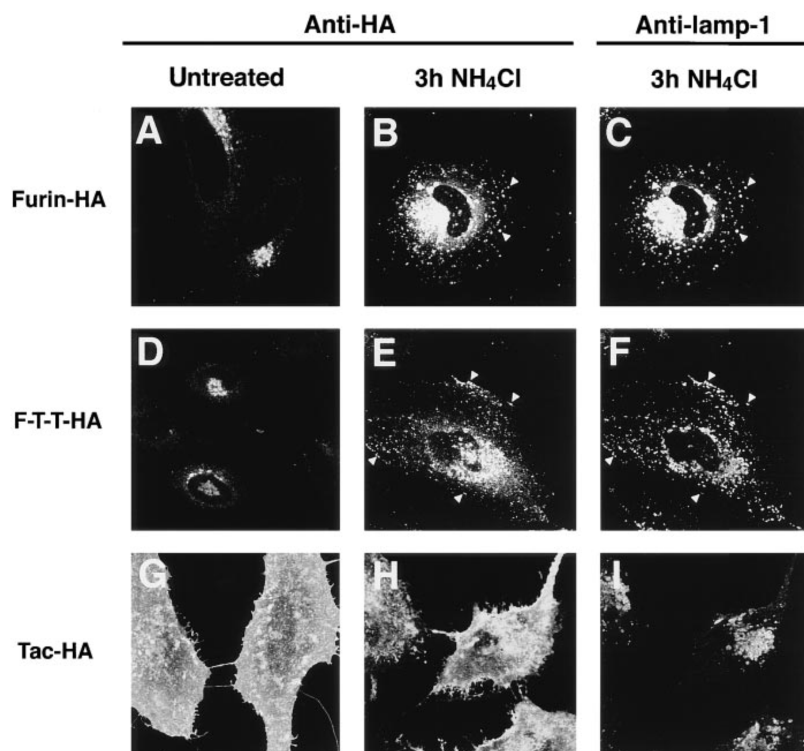

Figure 5. The luminal domain of furin mediates targeting to vesicles containing the lysosomal membrane protein lamp-1. Transiently transfected HeLa cells expressing furin-HA, Tac-HA, or F-T-T-HA were incubated for 3 h in the absence or presence of 50 mM NH_4Cl. Cells were fixed, permeabilized, and then stained with anti-HA and anti–lamp-1 antibodies, and a mixture of tetramethyl rhodamine anti–mouse IgG and fluorescein anti–rabbit IgG. Cells were examined by confocal microscopy as described in Materials and Methods. Notice the localization of furin-HA and F-T-T-HA to vesicles containing lamp-1 in NH_4Cl-treated cells (*arrowheads* in *B*, *C*, *E*, and *F*).

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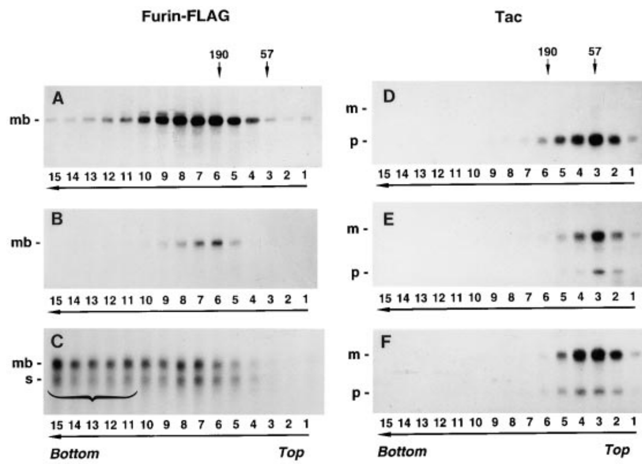

Figure 6. Aggregation of furin-FLAG in NH_4Cl-treated cells. Stably transfected RBL cells expressing furin-FLAG, Tac, or Tac-DKQTLL were pulse labeled for 30 min with [^{35}S]methionine and chased for 0 h (*A*, *D*, and *G*), 4 h (*B*), or 6 h (*C*, *E*, *F*, *H*, and *I*) in the absence (*A*, *B*, *D*, *E*, *G*, and *H*) or presence (*C*, *F*, and *I*) of 50 mM NH_4Cl. Detergent lysates of the cells were fractionated by sedimentation on 5–20% sucrose gradients for 16 h (see Materials and Methods). Individual gradient fractions (1–15) were immunoprecipitated with either an antifurin antibody (Fur2) (*A*–*C*) or an antibody to Tac (7G7) (*D*–*I*), and then analyzed by SDS-PAGE and fluorography. The MHC class I H-2K^b/β_2-microglobulin complex (~57 kD, fraction *3*) and the transferrin receptor (~190 kD, fractions *5* and *6*) were used as internal size standards for integral membrane proteins. Gradient fraction numbers, from top to bottom, are indicated. The bracket in *C* indicates high molecular weight aggregates of furin-FLAG.

dient used in this study was optimized for detecting aggregates and not for obtaining accurate measurements of the size of small oligomers.]) (Fig. 6 *A*). A more accurate estimation of the size of newly synthesized furin was obtained by chemical cross-linking with DTSSP, an agent having a cleavable disulfide bridge (Fig. 7). Substantial fractions of pulse-labeled furin-HA (Fig. 7 *A*, *arrow*) and furin-FLAG (Fig. 7 *B*, *arrow*) were cross-linked into an ~200-kD species, suggesting that newly synthesized ~100-kD furin polypeptide assembles into a homodimer. Only a fraction of furin-FLAG survived after a 4-h chase (Fig. 6 *B*); its size, as estimated by sedimentation velocity analysis, was similar to that observed after the pulse (Fig. 6 *A*). Strikingly, chasing in the presence of 50 mM NH_4Cl, resulted in accumulation of high molecular weight aggregates migrating all the way to the bottom of the gradient (Fig. 6 *C*, *bracket*). Both the ~100-kD membrane-bound form of furin and an ~80-kD soluble, luminal form of furin that accumulates in the presence of 50 mM NH_4Cl (Bosshart et

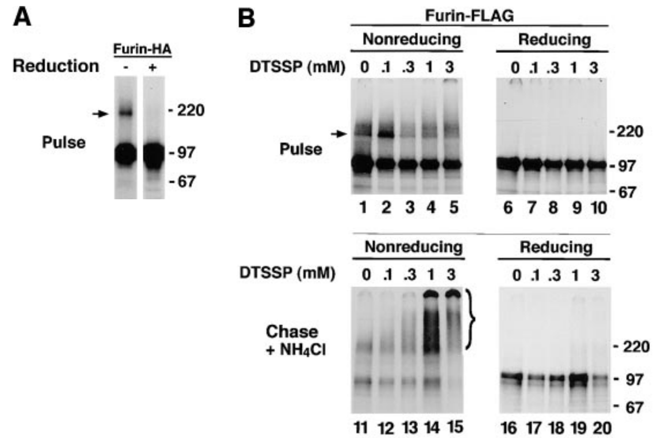

Figure 7. Analysis of the aggregation state of furin by chemical cross-linking. RBL cells expressing either furin-HA (*A*) or furin-FLAG (*B*) were pulse labeled with [^{35}S]methionine for 30 min at 37°C. Cells were either lysed immediately or chased for 4 h at 37°C in the presence of 50 mM NH_4Cl. Lysates were cross-linked with either 0.1 mM (*A*) or varying concentrations (*B*) of DTSSP. Furin-HA (*A*) was isolated by immunoprecipitation with an antibody mixture containing HA-11, Fur1, and Fur2. Furin-FLAG (*B*) was immunoprecipitated with M2, Fur1, and Fur2. Samples were resolved by SDS-PAGE under nonreducing or reducing conditions, as indicated in the figure. Arrows point to the position of the furin dimer. The positions of molecular mass markers (expressed as $10^{-3} \times M_r$) are shown at right.

al., 1994) were similarly aggregated. The accumulation of high molecular weight aggregates of furin-FLAG after a 4-h chase in the presence of 50 mM NH_4Cl could also be detected by cross-linking with DTSSP (Fig. 7 *B*, *bracket*). Some of the cross-linked aggregates were so large that they remained at the top of the stacking gel under nonreducing conditions (Fig. 7 *B*).

Both furin-FLAG and untagged furin expressed in HeLa cells were similarly aggregated after a 6-h chase in the presence of NH_4Cl (Fig. 8), suggesting that aggregation was not restricted to RBL cells, nor was it caused by the epitope tags. In addition, we observed that chasing in the presence of a mixture of leupepin, E64, pepstatin A and methionine methyl ester (LPEM), each of which inhibits lysosomal degradation by a mechanism distinct from that of NH_4Cl (Chen et al., 1988; Bosshart et al., 1994), also resulted in accumulation of furin-FLAG and furin-HA aggregates (Fig. 9). Thus, furin aggregation is not caused by NH_4Cl but rather revealed by inhibition of lysosomal degradation, regardless of the inhibitor used.

In contrast to furin-FLAG, Tac, which is transported to the plasma membrane and is relatively stable, did not change its size during chase either in the absence or the presence of NH_4Cl (Fig. 6, *D*–*F*). Similarly, Tac-DKQTLL, which is targeted to lysosomes by virtue of a di-leucine signal in the cytosolic domain (Letourneur and Klausner, 1992), did not undergo aggregation either in the absence or the presence of NH_4Cl in the chase medium (Fig. 6, *G*–*I*). From these results, we concluded that inhibition of lysosomal degradation results in the accumulation specifically of furin aggregates and not of proteins like Tac and Tac-DKQTLL, which are sorted by mechanisms unrelated to aggregation.

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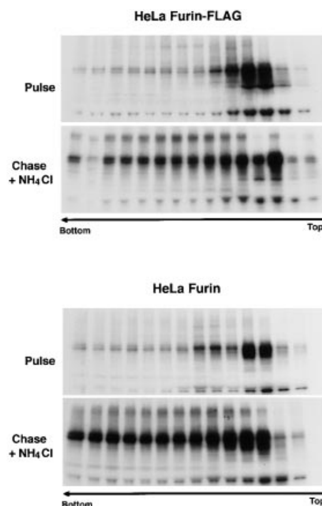

Figure 8. Both furin-FLAG and untagged furin aggregate in HeLa cells. HeLa cells were transiently transfected with plasmids encoding either furin-FLAG or untagged furin. Cells were labeled for 30 min with [^{35}S]methionine and chased for 6 h in the presence or absence of 50 mM NH_4Cl. The aggregation state of furin was assessed by sedimentation velocity analysis as described in the legend to Fig. 6.

Aggregation of Furin Is Mediated by Its Luminal Domain

To elucidate the domain(s) of furin required for aggregation, we analyzed the aggregation state of various furin and Tac chimeric proteins when lysosomal degradation was inhibited with the LPEM mixture (Fig. 9). We observed that all the proteins that contained the furin luminal domain became aggregated (furin-FLAG, furin-HA, furinΔ746-HA, and F-T-T-HA), whereas those that had the Tac luminal domain (Tac, T-T-F, T-F-F, T-T-F-HA, T-T-FΔ746-HA, and Tac-HA; see Fig. 1 for structures) did not (Fig. 9). From these experiments, we concluded that, like degradation, the aggregation of furin observed upon inhibition of lysosomal proteolysis was a property of the luminal domain of the protein.

Evidence That Aggregation of Furin Occurs within the TGN

To determine whether the aggregation of furin occurred within the TGN—thus preceding delivery of the protein to lysosomes—or after exit from the TGN, we performed temperature and pharmacologic manipulations that have been previously used to examine TGN-mediated processes. Incubation of cells at 19–20°C has been shown to arrest the trafficking of newly synthesized proteins at the level of the TGN (Griffiths et al., 1985; Sariola et al., 1995). Pulse-chase analyses showed that furin-FLAG was not detectably degraded over a 6-h chase at 19°C (Fig. 10 *A*, compare with chase at 37°C in Fig. 2), which is consistent with blockage of transport to lysosomes. Interestingly, cross-linking with DTSSP of extracts from cells chased at 19°C demonstrated absence of any high molecular weight aggregates of furin (Fig. 10 *B*, lane *1*) suggesting that aggregation itself was temperature dependent.

Temperature manipulations were then combined with the use of AlF_4^-. AlF_4^- activates GTP-binding proteins involved in the recruitment of organellar coats to membranes and inhibits various trafficking events by preventing the normal cycling of coat proteins between membrane and cytosolic pools (Donaldson et al., 1992; Carter et al., 1993; Finazzi et al., 1994). One of the trafficking steps inhibited by AlF_4^- is exit from the TGN (Kantanen et al., 1995; Sariola et al., 1995). In an immunofluorescence microscopy experiment similar to that shown in Fig. 5, we verified that AlF_4^- blocked transport of furin-HA from the TGN to a lysosomal compartment in chloroquine-treated cells at 37°C (data not shown).

We then analyzed the effects of AlF_4^- on degradation and aggregation of furin. We observed that raising the temperature from 19°C to 37°C in the absence of AlF_4^- resulted in time-dependent degradation of furin-FLAG that had accumulated in the TGN (Fig. 10 *B*, lanes *7–9*). This was likely because of resumption of transport to lysosomes at 37°C. In contrast, when the temperature shift was performed in the presence of AlF_4^-, furin was not degraded over a 3-h period (Fig. 10 *B*, lanes *10–12*), as would be expected from the AlF_4^--induced blockage of exit from the TGN. Analysis by DTSSP cross-linking showed that the

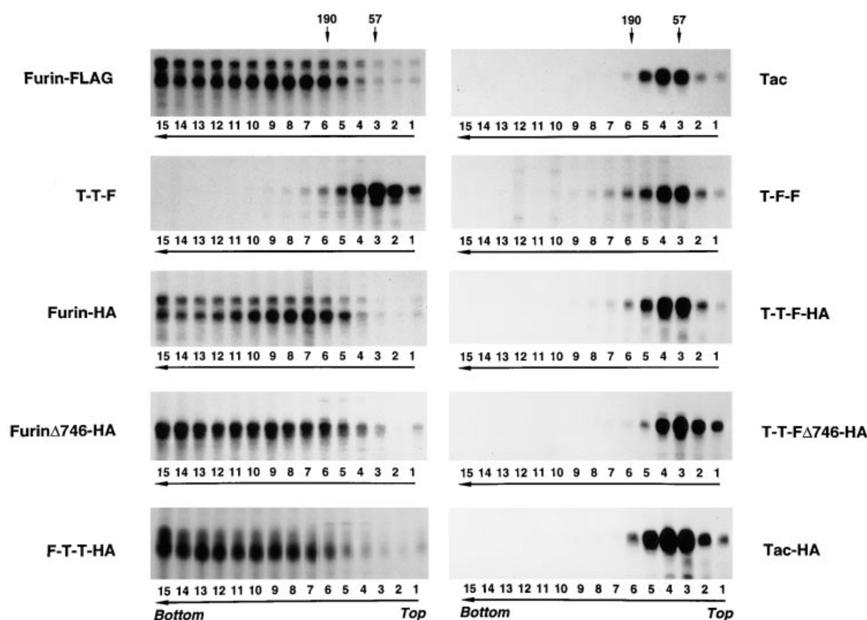

Figure 9. The luminal domain mediates aggregation of furin. Stably transfected RBL cells expressing the furin or Tac chimeras indicated in the figure were pulse labeled for 30 min with [^{35}S]methionine and chased for 4 h in the presence of a mixture of nonacidotropic lysosomal inhibitors (LPEM). Detergent-solubilized cells were fractionated by sedimentation on sucrose gradients and Tac and furin species were immunoprecipitated from the fractions as described in the legend to Fig. 6 and in Materials and Methods. Notice that only proteins having the furin luminal domain underwent aggregation during chase in the presence of lysosomal inhibitors.

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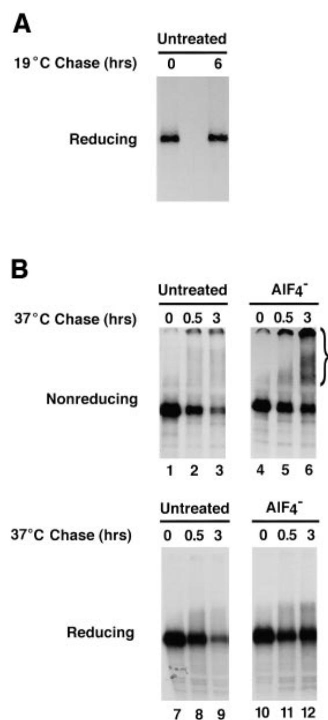

Figure 10. Effects of AlF_4^- on aggregation and degradation of furin accumulated in the TGN. (*A*) RBL cells expressing furin-FLAG were pulse labeled with [^{35}S]methionine for 30 min and chased for either 0 or 6 h at 19°C. Furin-FLAG was isolated by immunoprecipitation with a mixture of the antibodies M2, Fur1, and Fur2. Immunoprecipitates were resolved by SDS-PAGE. (*B*) RBL cells expressing furin-FLAG were pulse labeled with [^{35}S]methionine for 30 min and chased for 6 h at 19°C. Cells were then further chased for 30 min at 19°C and 6 h at 37°C in the absence (*Untreated*) or presence of aluminum fluoride (*AlF_4^-*). Cell lysates were cross-linked with 1 mM DTSSP and furin-FLAG was immunoprecipitated and analyzed as described in *A*.

furin that was protected from degradation by treatment with AlF_4^- existed as high molecular weight aggregates (Fig. 10 *B*, lanes *4–6*, *bracket*). On the basis of these observations, we concluded that the aggregation of furin occurs most likely in the TGN and thus precedes delivery of the protein to lysosomes.

Discussion

Furin localizes to the TGN by virtue of specific sorting information contained mainly within its cytosolic domain (Bosshart et al., 1994; Chapman and Munro, 1994; Molloy et al., 1994; Jones et al., 1995; Schäfer et al., 1995; Takahashi et al., 1995; Voorhees et al., 1995). In this study, we present evidence for a role of the luminal domain of furin in targeting to lysosomes for degradation. In addition, we show that inhibition of lysosomal degradation by various pharmacologic agents results in accumulation of aggregated furin in lysosomes. Like the degradation of furin, aggregation is also mediated by the luminal domain of the protein. These observations establish a strong correlation between aggregation and lysosomal targeting (see Fig. 1) and suggest that the two phenomena may be linked. Temperature and pharmacologic manipulations of transport from the TGN suggest that aggregation of furin occurs within the TGN, and precedes its transport to lysosomes. Thus, furin may become progressively aggregated during its residence in the TGN; aggregated furin would then be transported to lysosomes where the protein would be rapidly degraded.

Possible Significance of the Rapid Turnover of Furin

Most endogenous integral membrane proteins in the late secretory and endocytic pathways are degraded by the lysosomal system, although their rates of degradation vary widely. At one end of the range are lysosomal membrane proteins that, despite their localization to lysosomes, exhibit half-lives of several days (Lewis et al., 1985; Barriocanal et al., 1986; Green et al., 1987). At the other end are escort proteins such as the class II MHC-associated invariant chain (Blum and Cresswell, 1988) and the low-density lipoprotein receptor family–associated protein (RAP) (Czekay et al., 1997), which are degraded in late endosomal/lysosomal compartments soon after passage through the TGN. Rapid lysosomal turnover is not limited to escort proteins but is also a characteristic of cytokine receptors such as the erythropoietin receptor, which is constitutively removed from the plasma and degraded in lysosomes with a $t_{1/2}$ of <1 h (Neumann et al., 1993). The half-life of furin is therefore longer than that of cytokine receptors but shorter than that of other Golgi and TGN proteins, including TGN38 (originally known as $GIMP_t$), which has a $t_{1/2}$ of ~8 h (Yuan et al., 1987).

A previous study had shown that epitope-tagged forms of furin expressed in RBL cells had a $t_{1/2}$ = 2–4 h (Bosshart et al., 1994). Here, we demonstrate that untagged furin expressed in HeLa cells has a similarly short half-life, thus ruling out that the rapid turnover of furin is caused by addition of epitope tags or by its expression in RBL cells. Since endogenous furin is expressed at very low levels in most cells (van Duijnhoven et al., 1992; Shapiro et al., 1997), it is possible that the aggregation and degradation of the protein observed in this study are because of overexpression. However, the stably transfected RBL cells used in our experiments were selected for moderate expression levels. Furthermore, other proteins expressed at similar levels in RBL cells (e.g., all the Tac constructs) do not undergo aggregation after transport through the Golgi complex, and are degraded only if they have a specific lysosomal targeting signal (e.g., Tac-DKQTLL). Moreover, aggregation and degradation of furin-FLAG were observed in a stably transfected MDCK cell clone that expresses barely detectable levels of the protein (data not shown). These observations suggest that the aggregation and rapid turnover of furin are intrinsic properties of the protein.

The physiological significance of the rapid turnover of furin is unclear. Rapidly turning over proteins often fulfill regulatory functions. In addition to its role as a pro-protein convertase, furin has recently been implicated in the control of cell growth and differentiation (Konda et al., 1997), processes that may require rapid and precise attenuation of furin function. Targeting furin for lysosomal degradation would provide a mechanism for rapid lowering of protein levels under certain growth conditions. Another possibility is that the rapid turnover of furin is a consequence of its targeting to late endosomal compartments, where it might participate in endoproteolytic processing reactions. For example, furin could be involved in processing antigens for presentation by class II MHC molecules or other proteins that are targeted to late endosomal compartments before they are released into the medium. If lysosomal degradation results from a functional requirement to target furin to late endosomal compartments, it is not clear why the protein would not have a "lysosomal avoidance" signal such as that described for the cation-

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dependent, mannose 6-phosphate receptor (Rohrer et al., 1995), a protein with which furin shares some trafficking pathways. Finally, another possibility that has to be considered is that the aggregation and lysosomal targeting of furin in transfected cells is because of the absence of a specific "chaperone-like" molecule that stabilizes furin in the TGN.

Post-Golgi Aggregation As a Determinant of Lysosomal Targeting

The localization of most integral membrane proteins to organelles of the secretory and endocytic pathways can be explained on the basis of two fundamental mechanisms. The first mechanism relies on the recognition of discrete sorting signals by specific receptorlike molecules, as is the case for tyrosine-based sorting signals that target proteins to compartments of the endosomal/lysosomal system (Mellman, 1996; Marks et al., 1997). The second mechanism depends on a general physical-chemical property of the proteins that leads to retention in certain cellular environments. An example of this type of mechanism is the retention of some proteins in the ER and *cis*-Golgi cisternae, which may be mediated by formation of large aggregates (Rose and Doms, 1988; Hurtley and Helenius, 1989; Weisz et al., 1993). Both types of mechanism appear to contribute to the trafficking of furin within cells. Whereas the existence of specific sorting signals in the cytosolic domain of furin is now well established (Bosshart et al., 1994; Molloy et al., 1994; Jones et al., 1995; Schäfer et al., 1995; Takahashi et al., 1995; Voorhees et al., 1995), the results of the present study suggest that changes in the aggregation state of the furin luminal domain also contribute to the overall distribution and fate of the protein within cells. Thus furin is an example of a protein for which the concerted action of cytosolic and luminal determinants defines a complex pattern of protein trafficking.

Indirect evidence linking protein aggregation in the biosynthetic pathway with lysosomal targeting has been obtained for other proteins. For example, acidification in vitro was shown to cause aggregation of class II MHC molecules in the absence of bound antigenic peptides; this phenomenon was proposed to be the basis for the lysosomal degradation of unoccupied class II MHC molecules (Germain and Rinker, 1993). Another study demonstrated that treatment with leupeptin caused accumulation of aggregated class II MHC molecules in a lysosomal compartment (Amigorena et al., 1995). A more common observation is enhanced internalization and lysosomal targeting induced by cross-linking of cell surface molecules with antibodies or multivalent ligands (Ukkonen et al., 1986; Weissman et al., 1986). The lysosomal targeting of furin may thus be a manifestation of a general mechanism that diverts aggregated proteins from both biosynthetic and endocytic pathways to lysosomes. An attractive possibility is that this pathway provides a final level of quality control for proteins that become aggregated or otherwise damaged in post-Golgi compartments, in a manner analogous to ER quality control mechanisms (Bonifacino and Klausner, 1994; Hammond and Helenius, 1995).

Possible Mechanisms of Furin Aggregation

We have shown that furin appears to be a homodimer soon after synthesis in the ER (Figs. 6 *A* and Fig. 7). The fraction of the initial furin that remains after 4–6 h also behaves as a homodimer (Fig. 6 *B*). Aggregated furin, on the other hand, belongs to a population of molecules that are targeted for lysosomal degradation and can only be revealed by treatment with lysosomal inhibitors. We think that furin aggregates are most likely homo-aggregates; this idea is based on the absence of any other major proteins in furin immunoprecipitates, even after long-term labeling (data not shown). Unlike some aggregates of misfolded proteins formed in the ER (de Silva et al., 1993), furin aggregates are not disulfide bonded since they can be readily dissociated by SDS under nonreducing conditions (Fig. 7).

We speculate that aggregation could be triggered by a conformational change induced by exposure of furin to the environment of the TGN. Indeed, we have observed that recognition of furin by certain antibodies to luminal domain epitopes (e.g., Fur1 and Fur2) increases upon chase in the presence of lysosomal inhibitors (unpublished observations). This is why we used a mixture of antibodies to luminal and cytosolic epitopes in all of our immunoprecipitation experiments. An important event that takes place in the TGN is the dissociation of the furin pro-region fragment (Anderson et al., 1997); we speculate that this dissociation could render the protein more prone to aggregation. The ionic environment of the TGN could also contribute to the aggregation of furin. Several studies have shown that various proteins targeted to secretory granules aggregate at the high Ca^{+2} concentration and mildly acidic pH of the TGN (Freedman and Scheele, 1993; Shennan et al., 1994; Song and Fricker, 1995*b*; Colomer et al., 1996). This aggregation has been proposed to be an integral part of the mechanism by which some proteins are targeted from the TGN to the regulated secretory pathway (Bauerfeind and Huttner, 1993). It is thus conceivable that aggregation of furin may occur by a similar mechanism, and that its degradation may reflect transport to secretory granules that contain lysosomal proteins, as is the case for RBL cells (Bonifacino et al., 1989), or alternative transport to lysosomes in cells that lack regulated secretion (e.g., HeLa cells). The presence of furin in both mature and immature secretory granules has been reported (Song and Fricker, 1995*a*; Dittié et al., 1997), and it is possible that aggregation may play a role in sorting furin to these organelles. Finally, it has been speculated that a luminal cysteine-rich domain adjacent to the transmembrane domain of furin and other members of the furin family may play a role in sorting (Nakagawa et al., 1993). It would therefore be of interest to investigate the role of this domain in aggregation and degradation.

Concluding Remarks

Our studies of the intracellular trafficking of furin have uncovered an unusual phenomenon, namely, that the protein becomes aggregated upon transport into the TGN. Aggregation appears to promote targeting of the protein for lysosomal degradation, since the presence of the aggregated protein can only be evidenced by treatment with lysosomal inhibitors. Whereas the aggregation of furin is extensive and causes a profound alteration in the fate of the protein, we expect that more subtle changes in the aggre-

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gation state of other integral membrane proteins will also play a role in regulating their transport through secretory and endocytic routes.

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