The Cytoplasmic Domain Mediates Localization of Furin to the trans-Golgi Network En Route to the Endosomal/Lysosomal System

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Abstract. To investigate the mechanisms of membrane protein localization to the Golgi complex, we have examined the intracellular trafficking of epitope-tagged forms of the mammalian endopeptidase, furin, in stably transformed rat basophilic leukemia cells. Our studies show that furin is predominantly localized to the trans-Golgi network (TGN) at steady state, with smaller amounts present in intracellular vesicles. Biochemical and morphological analyses reveal that furin is progressively delivered to a lysosomal compartment, where it is degraded. Analyses of furin deletion mutants and chimeric proteins show that the cytoplasmic domain is both necessary and sufficient for localization to the TGN in various cell types. Interestingly, deletion of most of the cytoplasmic domain of furin results in a molecule that is predominantly localized to intracellular vesicles, some of which display characteristics of lysosomes. To a lesser extent, the cytoplasmically deleted molecule is also localized to the plasma membrane. These observations suggest the existence of an additional determinant for targeting to the endosomal/lysosomal system within the luminal and/or transmembrane domains of furin. Thus, the overall pattern of trafficking and steady state localization of furin are determined by targeting information contained within more than one region of the molecule.

The TGN is a compartment of the secretory pathway situated next to the trans face of the Golgi stack (reviewed by Griffiths and Simons, 1986; Geuze and Morré, 1991; Mellman and Simons, 1992). In mammalian cells, the TGN appears as a collection of tubules, cisternae, and vesicles, some of which are covered with clathrin coats (Geuze et al., 1984; Roth et al., 1985; Rambourg and Clermont, 1990). Within the TGN, newly synthesized proteins undergo terminal modification of their polypeptide and/or carbohydrate chains, including proteolytic cleavage and addition of sialic acid and sulfate groups (reviewed by Mellman and Simons, 1992). The TGN is also believed to be a major site of protein sorting within the secretory pathway. Newly synthesized proteins delivered to the TGN from the Golgi stack are sorted to various cellular compartments, including different domains of the plasma membrane, lysosomes, endosomes, and regulated secretory granules. The TGN also receives an influx of proteins from the plasma membrane and endosomal compartments, which are sorted along the same routes followed by newly synthesized proteins.

How the TGN is capable of performing all of these complex functions while maintaining its characteristic protein composition and structure is poorly understood. We have been particularly interested in the mechanisms by which certain proteins become predominantly localized to the TGN. One set of proteins found in the TGN includes carbohydrate-modifying enzymes, such as α2,6-sialyltransferase, which are also typical components of the Golgi stack (Roth et al., 1985). The presence of these proteins within the TGN probably reflects a gradual transition from the stack environment to that of the TGN. The primary determinant for Golgi localization of these proteins is found within the transmembrane domains (reviewed by Machamer, 1993). More recent observations have suggested that the TGN harbors another set of proteins that, while predominantly concentrated in the TGN, can also travel to more distal compartments of the secretory pathway. To date, the only well-characterized member of this set is the glycoprotein, TGN38/41 (Luzio et al., 1990; Reaves et al., 1992).

TGN38/41 is a type I integral membrane glycoprotein that exists as two isoforms, the sequences of which diverge near the carboxy terminus (Luzio et al., 1990; Reaves et al., 1992).
At steady state, the majority of TGN38/41 is found within the TGN, although smaller amounts are also present at the plasma membrane and in endosomal vesicles (Ladinsky and Howell, 1992; Bos et al., 1993). This distribution pattern is unusual among transmembrane proteins and reflects the existence of a cycling pathway between the TGN and the plasma membrane (Ladinsky and Howell, 1992; Reaves et al., 1993; Bos et al., 1993). Molecular dissection of TGN38 has implicated the cytoplasmic domain of the protein in mediating localization to the TGN (Luzio et al., 1990; Humphrey et al., 1993; Bos et al., 1993; Wong and Hong, 1993). Several groups have identified a tyrosine-containing segment of the TGN38 cytoplasmic domain that can confer TGN localization when placed within the cytoplasmic domain of various plasma membrane proteins (Humphrey et al., 1993; Bos et al., 1993; Wong and Hong, 1993). While the mechanism by which this sequence can confer TGN localization is unknown, its similarity to endocytosis signals (Trowbridge et al., 1993) has suggested a possible role in retrieval from a post-TGN compartment such as the plasma membrane or endosomes (Humphrey et al., 1993; Luzio and Banting, 1993; Stanley and Howell, 1993). To what extent the distribution, trafficking, and targeting information of TGN38/41 are characteristic of other TGN proteins remains to be established.

To further investigate the mechanisms of protein localization to the TGN, we decided to extend our studies to the intracellular trafficking of the mammalian endopeptidase, furin. Furin is a membrane-bound, dibasic endopeptidase predominantly localized to a late compartment of the Golgi system; this compartment has recently been identified as the TGN by Molloy et al. (1994). Furin is largely responsible for the proteolytic processing function that has long been known to exist within the Golgi complex (Bresnahan et al., 1990; Hatsuzawa et al., 1992; Misumi et al., 1991; Rehemtulla et al., 1992; Xu and Shields, 1993). On the basis of its membrane topology, furin is classified as a type I integral membrane glycoprotein having a large amino-terminal luminal domain, a single membrane-spanning sequence, and a carboxy-terminal cytoplasmic domain (see Fig. 1, scheme).

In the present study, we have examined the intracellular localization, trafficking and fate of epitope-tagged furin constructs in stably transfected rat basophilic leukemia (RBL)1 cells. The results of our studies show that while furin is predominantly localized to the TGN, a fraction of the molecules localizes to a vesicular compartment in these cells. Treatment with lysosomotropic agents increases detection of furin in vesicles that also contain the lysosomal integral membrane protein, lgp20. Pulse-chase analyses show that furin has a relatively short half-life in these cells and that its half-life is prolonged by treatment with inhibitors of lysosomal degradation. Taken together, these observations suggest that furin localization to the TGN in these cells is the result of a dynamic process that allows steady egress towards a prelysosomal or lysosomal compartment. Analyses of the intracellular localization of furin mutants and chimeric proteins show that the furin cytoplasmic domain is both necessary and sufficient for TGN localization. Interestingly, deletion of the cytoplasmic domain of furin results in increased delivery to lysosomal vesicles, in addition to a slight increase in expression at the plasma membrane. This observation suggests that an additional determinant present within the luminal and/or transmembrane domains of the molecule mediates its transport to the endosomal/lysosomal system. Furin may thus be an example of a protein for which a combination of targeting signals determines its overall pattern of localization and trafficking within the late secretory pathway.

Materials and Methods

Recombinant DNA Procedures

A cDNA clone encoding mouse furin (Hatsuzawa et al., 1990) was kindly provided by Dr. Cornelia Gorman (Genentech, South San Francisco, CA). Sequences encoding the FLAG (Hopp et al., 1988) and HA (Wilson et al., 1984) epitopes (see Table I for sequences) were fused in frame to the 3' end of furin coding sequences, using the PCR (Saiki et al., 1988). The resulting furin-FLAG and furin-HA constructs (see Fig. 1, scheme) were cloned into a modified version of the mammalian expression vector pCDL-SRa (Takebe et al., 1988). Constructs encoding the Tac antigen (Leonard et al., 1984) with either the FLAG or HA epitopes at the carboxy terminus (see Fig. 7 for Tac-HA scheme) were also made by PCR and cloned into the mammalian expression vector, pCDM8 (Seed, 1987). DNA sequences encoding the luminal and transmembrane domains of the Tac antigen (residues 1-262 of the primary translation product) were fused in frame to either the 5' carboxy-terminal residues (737-793) or the 10-membrane proximal residues (737-746) of the furin cytoplasmic domain, using the double PCR method of Higuchi et al. (1998). These constructs, respectively named T-F and T-Fa746 (see Fig. 9, scheme), were cloned into pCDM8. Versions of T-F-F and T-T-Fa746 with an HA epitope at the carboxy terminus were also made by PCR and cloned into pCDM8. The sequence of all the recombinant constructs was confirmed by the dyeoxy chain-termination method.

Cells

RBL cells (clone 2H3) were kindly provided by Dr. Henry Metzger (National Institutes of Health, Bethesda, MD). Normal rat kidney (NRK) and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DME (Biofluids, Rockville, MD) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Stable transfection of RBL and NRK cells and transient transfection of NRK and HeLa cells were done essentially as described by Humphrey et al. (1993). Single cell clones of stably transfected RBL and NRK cells were isolated by limiting dilution, and screened by immunofluorescence microscopy. Only clones that expressed moderate levels of the transfected constructs were used in our studies. RBL lines expressing Tac or a Tac-DKQTL construct (Leteurmeur and Klaussner, 1992) were the kind gift of Dr. Richard Klausner (National Institutes of Health, Bethesda, MD).

Specific Antibodies

The primary antibodies used in our studies and their sources are described in Table I.

Immunofluorescence Microscopy

Stably or transiently transfected cells were grown to 70-80% confluence on glass coverslips. Cells were 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 PBS containing 0.2% saponin. Unbound antibodies were removed by rinsing in PBS, after which cells were further incubated for 30 min with fluorescein labeled secondary antibodies (obtained from Jackson ImmunoResearch, West Grove, PA) in 0.2% saponin/PBS. After rinsing once more in PBS, the coverslips were mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Samples were examined with a Zeiss inverted microscope equipped with a 63× lens (Carl Zeiss, Oberkochen, Germany). In some experiments (see Fig. 3), fluorescently stained cells were examined using a BioRad MRC-600 laser-scanning confocal microscope. The 488- and 568-nm lasers were tuned to appropriate emission wavelengths. Images were acquired and processed with a BioRad MRC-600 laser scanning confocal microscope using a BioRad MRC-600 software (BioRad, Hercules, CA) and Macintosh II computer. Immunofluorescence microscopy was performed on the following cell types: RBL cells (clone 2H3) were kindly provided by Dr. Henry Metzger (National Institutes of Health, Bethesda, MD); normal rat kidney (NRK) and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DME (Biofluids, Rockville, MD) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Stable transfection of RBL and NRK cells and transient transfection of NRK and HeLa cells were done essentially as described by Humphrey et al. (1993). Single cell clones of stably transfected RBL and NRK cells were isolated by limiting dilution, and screened by immunofluorescence microscopy. Only clones that expressed moderate levels of the transfected constructs were used in our studies. RBL lines expressing Tac or a Tac-DKQTL construct (Leteurmeur and Klaussner, 1992) were the kind gift of Dr. Richard Klausner (National Institutes of Health, Bethesda, MD).

1. Abbreviations used in this paper: MME, methionine methyl ester; NPHGE, nonequilibrium pH gel electrophoresis; NRK, normal rat kidney; RBL, rat basophilic leukemia.
Electron Microscopy

Electron microscopy of ultrathin frozen sections of stably transfected RBL cells expressing furin-HA or furin-FLAG was performed essentially as described previously (Peters et al., 1991; Humphrey et al., 1993). Furin-HA-expressing cells were fixed in 2% glutaraldehyde/0.1 M phosphate buffer (pH 7.4) and furin-FLAG–expressing cells were fixed in 4% formaldehyde, 0.2 M Hepes buffer (pH 7.4) for 2 h at 37°C. Cells were then rinsed in PBS containing 0.15 M glycine, and embedded in 10% gelatin. Gelatin blocks were incubated for 16 h in 2.3 M sucrose at 4°C, and frozen in liquid nitrogen. 80-nm-thin cryosections were made with a cryo-ultramicrotome (Reichert Ultracut S). Sections were incubated at room temperature in 1% of the denaturing detergents SDS or Sarcosyl (N-Lauroyl Sarcosine; Sigma Chemical Co., St. Louis, MO), plus 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% SDS, or Sarcosyl. The sections were incubated at room temperature in 1% of the denaturing detergents SDS or Sarcosyl for 30 min and then fixed in 2% glutaraldehyde for 2 h at 4°C, rinsed with PBS and incubated for 30 min at 37°C in methionine-free DME containing 2% dialyzed FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (labeling medium). Lysates were then cleared by centrifugation for 15 min at 15,000 g at 4°C. In some experiments, cells were lysed for 10 min at room temperature in 1% of the denaturing detergents SDS or Sarcosyl (N-Lauroyl Sarcosine, Sigma Chemical Co., St. Louis, MO), plus 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4, 20 μg/ml DNAse I (Bethesda Research Laboratories, Gaithersburg, MD). After filtering through 0.45-μm Millex-HA filters (Millipore Corp., Bedford, MA), the lysates were diluted with regular lysis buffer to a final concentration of 0.1% SDS or Sarcosyl. The cleared lysates were incubated for 2 h at 4°C with antibodies bound to protein A-Sepharose, except for M2 which was bound to protein G-Sepharose. The Sepharose beads were washed three times with 0.1% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4, and three times with PBS, after which they were boiled for 5 min in reducing SDS-PAGE sample buffer. Immunoprecipitated proteins were separated by SDS-PAGE on 8 or 10% acrylamide gels, and gels were processed for fluorography with 1 M sodium salicylate. For endo H treatment, the washed beads were incubated for 16 h at 37°C with 1,000 NEB U of endo H (New England Biolabs, Boston, MA) in 0.1 M sodium citrate, pH 6.0, containing 0.1% SDS. The reaction was stopped by adding an equal volume of 2X SDS-PAGE sample buffer and boiling for 5 min. For neuraminidase treatment, the beads were incubated for 16 h at 37°C with 20 μl neuraminidase from Clostridium perfringens (Sigma Chemical Co.) in 50 mM sodium acetate buffer, pH 5.5, containing 80 mM CaCl₂. The immunoprecipitates were boiled for 5 min, dried, and processed for nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE, as described by O’Farrell et al. (1977).

Table 1. Antibodies

| Name  | Antigen | Antigenic sequence | Antibody type          | Source                                      |
|-------|---------|--------------------|------------------------|---------------------------------------------|
| DC16  | Furin   | DDGIEKNHPDLAGNT    | (153-167)              | Rabbit polyclonal                           |
| fur2  | Furin   | TQMDNRHGTTC        | (187-198)              | Rabbit polyclonal                           |
| fur1  | Furin   | NQNEKIQVTDLRQKC    | (345-360)              | Rabbit polyclonal                           |
| Cfur  | Furin   | SDSEEDEGRGIERATIKDQ | (772-793)             | Rabbit polyclonal                           |
| M2    | FLAG    | DYKDDDDK           |                        | Mouse monoclonal                            |
| 12CA5 | HA      | YPYDVPDYA          |                        | Mouse monoclonal                            |
| HA-11 | HA      | YPYDVPDYA          |                        | Rabbit polyclonal                            |
| JH4   | TGN38   | KTNRPDTQRLESDEKC   | (156-169)              | Rabbit polyclonal                            |
| LY1C6 | lgp120  |                    |                        | Mouse monoclonal                            |
| Anti-LAMP-1 | LAMP-1 |              |                        | Rabbit polyclonal                            |
| 7G7   | Tac     |                    |                        | Mouse monoclonal                            |
| R3134 | Tac     |                    |                        | Rabbit polyclonal                            |

Cells were thawed at 4°C and lysed by treatment for 15 min at 4°C in 1% Triton X-100, 0.5% sodium deoxycholate, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4 (regular lysis buffer). Lysates were cleared by centrifugation for 15 min at 15,000 g at 4°C. In some experiments, cells were lysed for 10 min at room temperature in 1% of the denaturing detergents SDS or Sarcosyl (N-Lauroyl Sarcosine, Sigma Chemical Co., St. Louis, MO), plus 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4, 20 μg/ml DNAse I (Bethesda Research Laboratories, Gaithersburg, MD). After filtering through 0.45-μm Millex-HA filters (Millipore Corp., Bedford, MA), the lysates were diluted with regular lysis buffer to a final concentration of 0.1% SDS or Sarcosyl. The cleared lysates were incubated for 2 h at 4°C with antibodies bound to protein A-Sepharose, except for M2 which was bound to protein G-Sepharose. The Sepharose beads were washed three times with 0.1% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl, pH 7.4, and three times with PBS, after which they were boiled for 5 min in reducing SDS-PAGE sample buffer. Immunoprecipitated proteins were separated by SDS-PAGE on 8 or 10% acrylamide gels, and gels were processed for fluorography with 1 M sodium salicylate. For endo H treatment, the washed beads were incubated for 16 h at 37°C with 1,000 NEB U of endo H (New England Biolabs, Boston, MA) in 0.1 M sodium citrate, pH 6.0, containing 0.1% SDS. The reaction was stopped by adding an equal volume of 2X SDS-PAGE sample buffer and boiling for 5 min. For neuraminidase treatment, the beads were incubated for 16 h at 37°C with 20 μl neuraminidase from Clostridium perfringens (Sigma Chemical Co.) in 50 mM sodium acetate buffer, pH 5.5, containing 80 mM CaCl₂. The immunoprecipitates were boiled for 5 min, dried, and processed for nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE, as described by O’Farrell et al. (1977).
Results

Carboxy Terminally Tagged Forms of Furin Are Predominantly Localized to the TGN

Endogenous furin is normally expressed at very low levels in most cultured cells (van Duijnhoven et al., 1992), so that the protein is difficult to detect by immunochemical techniques. To study the intracellular localization, trafficking and fate of furin, it was therefore necessary to express it by transfection of cDNA clones into cultured cells. To facilitate immunochemical detection of furin in various assays, recombinant DNA procedures were used to append either one of two short epitopic sequences, known as “FLAG” (Hopp et al., 1988) and “HA” (Wilson et al., 1984), to the cytoplasmic carboxy terminus of the protein (Fig. 1, scheme). These epitopes do not encode any known targeting signal and, in practice, we have found that they have no effect on the distribution of the plasma membrane protein, Tac, when fused to the cytoplasmic end of the protein (for example see Fig. 7 G). The resulting furin-FLAG and furin-HA constructs were expressed by stable transfection into RBL cells or by transient transfection into various other cell lines. In addition to antibodies to the FLAG and HA epitopes, we used four other anti-peptide polyclonal antibodies to various lumenal and cytoplasmic sequences of furin (see Fig. 1, scheme). These polyclonal antibodies to furin were effective at immunoprecipitating the protein, but proved less suitable than thentag antibodies for techniques involving immunostaining of fixed cells.

Immunofluorescence microscopy of stably transfected RBL cells showed staining of a juxtanuclear structure characteristic of the Golgi complex for both furin-FLAG (Fig. 1 A) and furin-HA (Fig. 1 C). A similar pattern of staining was observed for the TGN-localized protein, TGN38 (Fig. 1, B and D), confirming the identity of this juxtanuclear structure as the Golgi complex. The localization of the two epitope-tagged furins was thus similar to that reported for untagged furin (Bresnahan et al., 1990, Misumi et al., 1991, van Duijnhoven et al., 1992), suggesting that fusion of either epitope at the carboxy terminus had no effect on the distribution of the molecule. Essentially the same pattern of staining was

![Image](image-url)

Figure 1. (Top) Schematic representation of epitope-tagged furin constructs used in this study. The scheme represents the primary translation products, before any proteolytic processing events. The number of amino acid residues in each portion of the molecule is indicated in parenthesis. The figure also depicts the approximate positions of the three sites of N-glycan addition and the locations of peptide sequences recognized by antibodies to furin (DC16, fur2, fur1, and Cfur), FLAG (M2), and HA (12CA5 or HA-11) (see Table I). (Bottom) Immunofluorescence microscopy localization of furin-FLAG and furin-HA in stably transfected RBL cells. RBL cells expressing furin-FLAG (A and B) or furin-HA (C and D) were fixed, permeabilized and stained simultaneously with: (A and B) a mouse antibody to the FLAG epitope (M2) and a rabbit antibody to TGN38 (JH4), or (C and D) a mouse antibody to the HA epitope (12CA5) and a rabbit antibody to TGN38 (JH4). The primary antibodies were revealed by incubation with a mixture of fluorescein-conjugated donkey antibodies to mouse IgG and Texas red-conjugated donkey antibodies to rabbit IgG. (A and C) Fluorescein channel; (B and D) Texas red channel. Control experiments showed no staining for FLAG or HA in untransfected RBL cells (not shown). Bar, 10 μm.
Figure 2. Immunoelectron microscopy localization of epitope-tagged furins in stably transfected RBL cells. Thin frozen sections of fixed RBL cells expressing either furin HA (A) or furin-FLAG (B and C) were stained with antibodies to the HA (HA-11) or FLAG (M2) epitopes, respectively, as described in the Materials and Methods section. The bound primary antibodies were detected with either 10-nm gold-conjugated protein A (A) or 5-nm gold-conjugated antibodies to mouse immunoglobulins (B and C). For both furin-HA (A) and furin-FLAG (not shown), most of the gold particles were found to be concentrated on tubules and vesicles in an area adjacent to the Golgi stack (g). Some of the positively stained structures in this area were found to be coated by an electron dense material characteristic of clathrin coats (B, arrowheads). The location and morphology of these structures are characteristic of the TGN. Some gold particles were detected inside vesicular structures (v) with the morphology of multivesicular bodies, such as the one shown in C. There was no significant staining of the Golgi stack or the plasma membrane (p). Bars: (A) 0.2 μm; (B and C) 0.1 μm.
observed upon transient expression of the proteins in NRK cells (for example see Fig. 7 A) and HeLa cells (not shown). Interestingly, some of the stably transfected RBL cells displayed additional staining of cytoplasmic vesicles, which could be best resolved by confocal microscopy (see Fig. 3 A).

The distribution of furin-HA and furin-FLAG in RBL cells was further analyzed by quantitative immunogold electron microscopy (Fig. 2). Both proteins were found to be predominantly localized to tubules and vesicles on one side of the Golgi stack (~75% of gold particles; see Fig. 2 A for example). This region of the cell was characterized by the presence of coated vesicles or coated buds, some of which contained epitope-tagged furin (Fig. 2 B). The characteristics of this structure clearly correspond to those described previously for the TGN (Griffiths and Simons, 1986; Rambourg and Clermont, 1990). These observations are in agreement with those of Molloy et al. (1994), who recently reported a similar localization for a lumenaUly tagged furin construct expressed using a vaccinia infection system. Taken together, these observations argue that furin resides predominantly within the TGN, and that its ability to localize to this compartment is unaffected by the addition of epitope tags at either end of the polypeptide chain.

Our immunoelectron microscopy analyses also revealed the presence of approximately 10% of furin-FLAG in vesicular structures, some of which had the appearance of multivesicular bodies (v, Fig. 2 C). Multivesicular bodies have been previously shown to correspond to late endosomal or prelysosomal compartments (Tougard et al., 1985; Jost-Vu et al., 1986; Griffiths et al., 1990). No significant deposition of gold particles was evident at the plasma membrane (Fig. 2 A), although more sensitive, flow cytofluorometry analyses

Figure 3. Effect of MME on the distribution of furin-FLAG in stably transfected BRL cells. RBL cells expressing furin-FLAG (A and B) or furin-HA (C and D) were incubated for 4 h in regular culture medium, in the absence (A) or presence (B–D) of 20 mM MME. Cells were then fixed, permeabilized, and prepared for immunofluorescence microscopy. Cells shown in A and B were stained with antibodies to the FLAG epitope (M2) and Texas red-conjugated donkey antibodies to mouse IgG. Cells in C and D were double-stained with rabbit antibodies to the HA epitope (HA-11) and mouse antibodies to Igpl20 (LYIC6d), followed by Texas red-conjugated donkey antibodies to rabbit IgG and fluorescein-conjugated donkey antibodies to mouse IgG. Slides were examined by confocal microscopy. C and D show the colocalization of furin-HA and Igpl20, respectively, in a region of an MME-treated cell. The degree of colocalization in other cells was also significant, but less extreme than in the example shown in C and D.
of furin-HA transfectants revealed the presence of a small but significant amount of furin at the cell surface (data not shown).

**Treatment with Lysosomotropic Agents Causes Accumulation of Epitope-tagged Furin in Lysosomal Vesicles**

The presence of some epitope-tagged furin molecules in what appeared to be endosomal or prelysosomal structures prompted us to investigate whether furin-FLAG was capable of leaving the TGN and moving into a post-TGN, potentially degradative compartment. To address this possibility, we examined the effect of the lysosomal inhibitor, methionine methyl ester (MME) (Reeves et al., 1981), on the distribution of epitope-tagged furin within cells. Treatment with MME led to a substantial increase in the number and intensity of cytoplasmic vesicles containing immunoreactive furin (Fig. 3 B). In these vesicles, furin was found to colocalize partially with the lysosomal integral membrane protein, Igpl20 (Fig. 3, C and D). This protein has been previously shown to be enriched within prelysosomal and lysosomal structures (Griffiths et al., 1988; Geuze et al., 1988). RBL cells have been shown to have an additional population of regulated secretory vesicles that contain both soluble and membrane-bound lysosomal proteins (Bonifacino et al., 1989). The vesicles stained in MME-treated cells could thus correspond to any or all of these Igpl20-containing structures. Since in this study we have attempted to distinguish between these different structures, we refer to them throughout the paper as "lysosomal vesicles" or, more generally, as a "lysosomal compartment". Other vesicles containing epitope-tagged furin but not Igpl20 could correspond to endosomes. The results of this experiment suggested that a significant amount of furin-FLAG could undergo transport from the TGN to lysosomal vesicles over the 4-h span of the experiment. We could not ascertain, however, whether the detection of furin in lysosomal vesicles was a manifestation of a normal transport process or whether it was caused by perturbation of the TGN or of membrane trafficking pathways by MME.

**Biosynthesis and Processing of Epitope-tagged Furins in RBL Cells**

To corroborate the results of the morphological analyses, and to obtain additional information on the trafficking and fate of furin within cells, we conducted a series of pulse-chase, metabolic labeling experiments. After a 30-min pulse with [35S]methionine, furin-FLAG appeared as an endo H-sensitive doublet of ~105,000 and ~95,000 M\(_r\) (Fig. 4 A, lanes 1 and 2). The higher relative molecular mass species probably corresponds to the newly synthesized zymogen form of the molecule, whereas the lower relative molecular mass species represents a proteolytically processed form lacking an amino terminal proregion sequence (Misumi et al., 1991; Molloy et al., 1994; Rehemtulla et al., 1992; Leduc et al., 1992). The rapid conversion of the zymogen to the processed form and the endo H-sensitive nature of both species are consistent with this cleavage occurring within the ER. Within 1 h of chase, most of the proteolytically processed furin shifted up in molecular weight (~100,000 M\(_r\)) and became resistant to endo H (Fig. 4 A, lanes 3 and 4). After a 2-h chase, virtually all of the labeled furin-FLAG was resistant to endo H (Fig. 4 A, lanes 5 and 6) (meaning that it has passed through the medial Golgi cisternae) but became sensitive to neuraminidase (Fig. 4 B) (indicating transport of the trans-Golgi cisternae and TGN). It is worth mentioning that the efficiency of processing of newly synthesized furin observed in our experiments with stably transfected RBL cells was markedly higher than that reported previously in transiently transfected COS cells (Misumi et al., 1991). Our observations thus demonstrated that furin-FLAG rapidly exited the ER and traversed the entire Golgi complex in RBL cells, in accordance with its localization to the TGN revealed by the immunoelectron microscopy studies.

**Evidence for Lysosomal Degradation of Epitope-tagged Furins**

More extended pulse-chase analyses showed that furin-FLAG disappeared from stably transfected RBL cells without being quantitatively recovered in the culture medium (Fig. 5). The half-time of disappearance of the protein was ~2 h in the experiment shown in Fig. 5, although it was
treated with a mixture of lysosomal inhibitors, including MME, leupeptin, pepstatin A, and E64 (Fig. 5, +LPEM). This treatment decreased the rate of loss of furin-FLAG and led to the intracellular accumulation of a ~80,000-Mr fragment (Fig. 5). Similar results were obtained in cells treated with 50 mM ammonium chloride (data not shown). Like furin-FLAG, furin-HA was also found to be degraded by a process sensitive to lysosomotropic agents (data not shown and see Fig. 8), suggesting that the fate of furin is independent of the sequence of the epitope tag. All of these observations were consistent with degradation of epitope-tagged furins occurring within a lysosomal compartment.

**Generation of a Soluble, Luminal Intermediate in the Degradation of Epitope-tagged Furin**

The ~80,000-Mr fragment generated intracellularly in ammonium chloride-treated cells could be immunoprecipitated with antibodies to luminal (e.g., Fig. 6, lane 1 fur2) but not cytoplasmic epitopes (e.g., Fig. 6, lane 2 M2). This observation indicated that the fragment lacked the carboxy-terminal, cytoplasmic end. To examine whether the ~80,000-Mr fragment was membrane-bound or soluble, ammonium chloride-treated cells were mechanically disrupted under conditions that cause breakage of lysosomes. Soluble and membrane-bound protein fractions were then analyzed for the presence of furin. As shown in Fig. 6 B, the ~80,000-Mr species was almost completely released into the supernatant during the initial homogenization (lane 3), in contrast to the ~100,000-Mr species, which remained bound to membranes even after treatment of the pellet with sodium carbonate (lanes 4 and 5). These observations suggest that furin undergoes a proteolytic cleavage that produces a soluble fragment comprising most of the luminal domain. This fragment may correspond to a transient intermediate in the normal degradation process and may also be similar to the soluble fragment that is normally released into the medium in small amounts.

**The Cytoplasmic Domain of Furin Is Necessary for Localization of the Protein to the TGN: Delivery of the Cytoplasmically Deleted Protein to a Lysosomal Compartment**

Having established that furin is predominantly localized to the TGN at steady state and eventually delivered to a lysosomal compartment for degradation, we decided to examine what topologic domains of the protein were involved in determining its localization and fate. Because of previous studies that implicated the cytoplasmic domain of TGN38 in TGN localization (Bos et al., 1993; Humphrey et al., 1993; Luzio et al., 1990; Wong and Hong, 1993), we focused on the same domain of furin. Recombinant constructs (Fig. 7, scheme) were expressed by transfection into RBL, NRK, and HeLa cells with similar results. Fig. 7 shows only the results obtained in NRK and HeLa cells, because these were easier to reproduce photographically. Removal of a 47-amino acid segment (residues 746-793) from the carboxy terminus of furin, in the context of furin-HA, resulted in a protein (furinΔ746-HA) that no longer localized to the TGN, but was instead predominantly found within a vesicular compartment in RBL (not shown), NRK (Fig. 7 C) and HeLa cells (Fig. 7 E). In all of these different cell types, we also noticed occasional staining of the cells’ edges (Fig. 7 C, arrowheads), which suggests that a fraction of the truncated

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**Figure 5. Analysis of the turnover of furin-FLAG.** RBL cells expressing furin-FLAG were metabolically labeled for 30 min with [35S]methionine and chased for the periods indicated in the figure, in the absence (Untreated) or presence (+LPEM) of a mixture of lysosomal inhibitors containing 20 mM MME and 100 μg/ml each of leupeptin, pepstatin A, and E64. Furin was isolated from both detergent-extracted cells and culture media, using antibodies to a luminal epitope (DC16). Immunoprecipitates were resolved by SDS-PAGE under reducing conditions on 8% acrylamide gels. The lower two panels (Medium, 30X) show a 30 times longer exposure of the middle panels (Medium). The positions of molecular weight markers (expressed as 10⁻³ × Mr) are shown at left.
protein was transported to the plasma membrane. The truncated furin present in vesicles was partially colocalized with a lysosomal membrane protein (Fig. 7, E and F, arrowheads), indicating that at least some of the vesicles correspond to lysosomal structures. Other vesicles may correspond to intermediates in the endosomal/lysosomal system. From these observations, we concluded that the cytoplasmic domain of furin is necessary for localization of the protein to the TGN.

The partial localization of furinΔ746-HA to lysosomal vesicles suggested that, in the absence of the TGN localization determinant, the protein might be directly or indirectly transported to a lysosomal compartment. If this were the case, one would expect to observe a more rapid degradation of the cytoplasmically deleted furin as compared to the full length protein. Indeed, pulse–chase analyses of stably transfected RBL cells showed that furinΔ746-HA had a shorter half life than furin-HA (Fig. 8). Degradation of furinΔ746-HA was inhibited by ammonium chloride (data not shown), indicating that it also took place in a lysosomal compartment. These findings suggest the possible existence of a determinant for lysosomal delivery within furin, that is distinct from cytoplasmic sequences involved in TGN localization.

The Cytoplasmic Domain of Furin Contains Sufficient Information for TGN Localization

To assess whether all the information required for TGN localization was contained within the cytoplasmic domain of furin, we constructed a chimeric protein having the luminal and transmembrane domains of the plasma membrane protein, Tac (Leonard et al., 1984), and the cytoplasmic domain of furin. This construct, denoted T-T-F, was found to colocalize with TGN38 in both transiently transected NRK cells (Fig. 9, C and D) and stably transfected RBL cells (not shown), unlike the normal Tac antigen which was expressed at the plasma membrane (Fig. 9 A). Immunoelectron microscopy of stably transfected NRK cells expressing T-T-F confirmed that the chimeric protein was predominantly found in the region of the TGN (Fig. 10, A–C), including numerous coated membrane structures (Fig. 10, arrowheads). Smaller amounts of the chimeric protein were observed in vesicular structures resembling endosomes or multivesicular bodies (Fig. 10 C). Similar observations were made in stably transfected RBL cells (not shown). Deletion from T-T-F of the same 47-amino acid segment previously examined in the context of furin, caused the truncated protein (T-T-FΔ746) to be transported to the plasma membrane, as observed by immunofluorescence microscopy (Fig. 9 E). This experiment demonstrated that the 10 membrane proximal amino acids of the furin cytoplasmic domain that remained in this construct have no additional sorting information. Placement of the HA epitope at the carboxy-termini of T-T-F and T-T-F-Δ476 had no effect on the localization of either chimeric protein (data not shown), thus demonstrating that no targeting signals are generated by fusion of the HA epitope to furin cytoplasmic sequences. On the basis of these observations, we concluded that the carboxy-terminal 47 amino acids of furin contain sufficient information for protein localization to the TGN.

To corroborate that the cytoplasmic domain of furin was not responsible for the lysosomal turnover of the protein, we also examined the stability of the T-T-F construct in stably transfected RBL cells. We observed that the chimera was only minimally degraded over a 4-h period, in contrast to a Tac construct having a lysosomal-targeting, di-leucine motif (Letourneur and Klausner, 1992), which was rapidly degraded in an ammonium chloride–dependent fashion (Fig. 11).

Taken together, the above findings suggest that the TGN localization of furin is mediated by a signal within its cytoplasmic domain, whereas its delivery to intracellular vesicles and eventual lysosomal degradation is directed by a determinant within the luminal and/or transmembrane domains.

Discussion

Intracellular Localization and Turnover of Furin

Our morphological and biochemical analyses of stably transfected RBL cells have revealed two salient features of the
Figure 7. The cytoplasmic domain of furin contains information necessary for protein localization to the TGN. (Top) Schematic representation of furin-HA, furinΔ746-HA, and Tac-HA. The number of amino acid residues in each region of the molecules are indicated in parenthesis. The lumenal domain of furin-HA and furinΔ746-HA corresponds to that of the zymogen form, after cleavage of the signal peptide but prior to removal of the amino terminal pro-region. Residues in the lumenal domain of Tac are counted from the initiating methionine.
trafficking of carboxy-terminally tagged forms of furin: (a) that the proteins are predominantly localized to the TGN at steady state; and (b) that they undergo a relatively rapid turnover involving delivery to a lysosomal compartment. These observations suggest that furin may reside within the TGN for a limited period of time, after which the protein is progressively targeted for lysosomal degradation.

While the fact that furin localizes to the TGN has been demonstrated in various cell types by us and others (Molloy et al., 1994), it is not yet known whether its rapid lysosomal turnover is a general phenomenon in all cells. This question is particularly relevant because of the fact that RBL cells, which do not have regulated secretory vesicles, do not accumulate at the cell surface but are predominantly localized to intracellular vesicles in various cell types. In HeLa cells, which do not have regulated secretory vesicles, furinΔ746-HA was also found in vesicles, some of which contained the lysosomal membrane protein, LAMP-1. Other vesicles that contained furinΔ746-HA but were negative for LAMP-1 could correspond to endosomal structures. Molloy et al. (1994) also observed localization of a cytoplasmically truncated furin to intracellular vesicles in BSC-40 cells, although the identity of such vesicles was not established.

Another caveat regarding the lysosomal targeting of furin constructs is to what extent the carboxy-terminal tags affect the trafficking of the molecules. It is indeed possible that the epitope tags reduce the efficiency with which furin is maintained in the TGN and/or cause increased delivery to the endosomal/lysosomal system. However, we think that it is unlikely that the tags are responsible for the eventual localization of cytoplasmically truncated furin to intracellular vesicles. This idea is based on the observations that neither the HA nor the FLAG tags affect the localization of the Tac antigen to the plasma membrane when placed at the carboxy terminus of the molecule. In addition, placement of the HA tag at the carboxy termini of T-T-F and T-F-FΔ746 has no effect on the localization of those constructs to the TGN and the plasma membrane, respectively.

**Dynamic Nature of Furin Localization within Cells**

The observed steady state localization of epitope-tagged furins to the TGN appears to be the result of a dynamic process involving transient maintenance in the TGN and then progressive delivery to intracellular vesicular compartments, including lysosomes. These features have been incorporated into a working model for furin trafficking depicted in Fig. 12 A. According to this model, newly synthesized furin rapidly traverses the Golgi stack and reaches the TGN. The protein is then either transiently retained within the TGN or engages in cycling between the TGN and a more distal compartment (e.g., an endosomal/pre-lysosomal compartment or the plasma membrane). A fraction of the furin molecules may be either released from TGN retention or diverted from the cycling pathway, and delivered to a lysosomal compartment for degradation. Routing of furin to a lyso-

![Figure 8](image-url)

Figure 8. Comparison of the turnover of furin-HA and furinΔ746-HA in stably transfected RBL cells. RBL cells expressing furin-HA or furinΔ746-HA were metabolically labeled for 30 min with [35S]methionine and chased in complete medium for the times indicated in the figure. Furin was then isolated form detergent-solubilized cells and from culture media using a mixture of polyclonal antibodies (DC16, fur2, furl, and HAll). Immunoprecipitates were resolved by SDS-PAGE under reducing conditions on 8% acrylamide gels. The positions of molecular weight markers (expressed as 10⁻³ × Mr) are shown to the left.

(Bottom) (A and B) Double immunofluorescence microscopy localization of furin-HA and TGN38 in transiently transfected NRK cells. (C and D) Double immunofluorescence microscopy localization of furinΔ746-HA and TGN38 in transiently transfected NRK cells. (E and F) Double immunofluorescence microscopy localization of furinΔ746-HA and LAMP-1 in transiently transfected HeLa cells. (G and H) Double immunofluorescence microscopy localization of Tac-HA and TGN38 in transiently transfected NRK cells. Cells were fixed, permeabilized, and stained simultaneously with a mouse antibody to the HA epitope (12CA5) (A, C, E, and G) and rabbit antibodies to endogenous TGN38 (B, D, and H) or LAMP-1 (F), followed by Texas red-conjugated donkey antibodies to mouse IgG and fluorescein-conjugated donkey antibodies to rabbit IgG. Notice the colocalization of furin-HA (A) with endogenous TGN38 (B) and the presence of furinΔ746-HA in vesicles (C). The arrowheads in C point to a cell expressing some furinΔ746-HA at the plasma membrane. Arrowheads in E and F indicate vesicles that contain both furinΔ746-HA and LAMP-1. Bar, 10 μm.

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Figure 9. The cytoplasmic domain of furin has sufficient information for protein localization to the TGN. (Top) Schematic representation of Tac and the Tac-furin chimeric proteins, T-T-F, and T-T-FΔ746. The number of amino acid residues in each topologic domain is indicated in parenthesis. Residues in the luminal domain of Tac are counted from the initiating methionine. (Bottom) Immunofluorescence localization of Tac and Tac-furin chimeric proteins in transiently transfected NRK cells. Transiently transfected NRK cells expressing Tac (A and B), T-T-F (C and D), or T-T-FΔ746 (E and F) were fixed, permeabilized and stained simultaneously with a mouse antibody to Tac (7G7) and rabbit antibodies to endogenous TGN38 (JH4), followed by Texas red-conjugated donkey antibodies to mouse IgG and fluorescein-conjugated donkey antibodies to rabbit IgG. (A, C, and E): Texas red channel, Tac staining; (B, D, and F): fluorescein channel, TGN38 staining. Notice the presence of normal Tac (A) and T-T-FΔ746 (E) at the plasma membrane and the colocalization of T-T-F (C) with endogenous TGN38 (D). Bar, 10 μm.
Figure 10. Immunoelectron microscopy localization of T-T-F in stably transfected NRK cells. Thin frozen sections of fixed NRK cells expressing T-T-F were incubated with a rabbit polyclonal antibody to Tac (R3134) and 10-nm gold-conjugated protein A, as described in the Materials and Methods section. Notice the deposition of most of the gold particles in the area of the TGN (A-C), including numerous coated membrane structures (B, arrowheads). A smaller fraction of the gold particles were observed in vesicular organelles (C). Bar, 0.2 μm.
Figure 11. The cytoplasmic domain of furin does not mediate rapid lysosomal degradation. RBL cells stably expressing the chimeric proteins T-T-F or Tac-DKQTLL (Letourneur and Klausner, 1992) were metabolically labeled for 30 min with [35S]methionine and chased for 4 h in the absence or presence of 50 mM ammonium chloride. Tac proteins were isolated from detergent-solubilized cells and culture media using antibodies to a Tac lumenal epitope (7G7). Immunoprecipitates were analyzed by reducing SDS-PAGE on 10% acrylamide gels. The positions of molecular weight markers (expressed as 10^{-3} \times M_r) are shown to the left.

Consistent with the latter hypothesis is the finding that a still undetermined but appreciable amount of furin is expressed at the cell surface (Molloy et al., 1994). As shown by our electron microscopy studies, the amount of furin present on the cell surface at any given time is likely to be small, although it can be detected by antibody binding followed by flow cytofluorometry (data not shown) or by antibody uptake (Molloy et al., 1994). Increased levels of surface staining were observed in cells treated with MME or ammonium chloride (data not shown) or upon deletion of part of the cytoplasmic domain (Fig. 7 C). These observations raise the possibility that, analogous to the trafficking of TGN38 (Bos et al., 1993; Reaves et al., 1993; Ladinsky and Howell, 1992), some or all of the furin molecules transit through the cell surface during their lifetime. Trafficking through the cell surface may be a requisite step for steady state localization to the TGN or for lysosomal delivery. In addition, cycling through the cell surface may fulfill a physiological requirement for furin activity in compartments of the secretory pathway other than the TGN, as previously suggested (Mizumii et al., 1991; Smeekens et al., 1992; Klimpel et al., 1993; Tsuneoka et al., 1993; Moehring et al., 1993).

Role of the Cytoplasmic Domain of Furin in TGN Localization

Our studies, and those of Molloy et al. (1994), have shown that deletion of the furin cytoplasmic domain causes a dramatic change in the steady state distribution of furin. The truncated furin no longer localizes to the TGN, but is delivered primarily to a vesicular compartment, and to a lesser extent to the plasma membrane (see model in Fig. 12 B). Like the cytoplasmic domain of TGN38 (Bos et al., 1993; Humphrey et al., 1993; Luzio et al., 1990; Wong and Hong, 1993), the cytoplasmic domain of furin thus plays a preeminent role in protein localization to the TGN. As previously hypothesized for TGN38 (Humphrey et al., 1993), the cyto-

Figure 12. Models for the intracellular trafficking of furin (A) and a cytoplasmically deleted furin construct (B). See Discussion for details.
plasmic domain of furin could be involved in causing transient retention within the TGN, or participate in retrieval from a post-TGN compartment.

Our studies also show that the furin cytoplasmic domain is not only necessary for furin localization to the TGN, but is also sufficient to confer such localization on two plasma membrane proteins, Tac (Figs. 9 and 10) and CD4 (not shown). This finding suggests the existence of an autonomous TGN localization signal within this domain, the nature of which remains to be established. In this regard, it is worth noting that there are several aromatic amino acid (tyrosine and phenylalanine) residues within this sequence (Hatsuzawa et al., 1990); this type of amino acid has been previously implicated in the localization of other proteins to late compartments of the Golgi system in both mammalian and yeast cells (Wilcox et al., 1992; Nothwehr et al., 1993; Humphrey et al., 1993; Bos et al., 1993; Wong and Hong, 1993). Aromatic amino acid–based sequences are the likely binding site of cytosolic proteins or protein complexes that participate in the mechanism of localization to the TGN. Since a cytoplasmically truncated furin is mostly retained intracellularly, albeit not in the TGN, it is likely that other regions of the molecule assist in determining the overall pattern of furin localization and movement within cells.

**Similarities in the Trafficking of Mammalian Furin and Its Yeast Homologue, Kex2p**

In many respects, mammalian furin behaves similarly to its yeast homologue, Kex2p. Like furin, Kex2p localizes to a late compartment of the Golgi complex, which is probably a functional equivalent of the mammalian TGN (Redding et al., 1991). Deletion of the cytoplasmic tail of Kex2p results in loss of late Golgi localization and direct transport to the vacuole for degradation (Wilcox et al., 1992). Two other late Golgi integral membrane proteins, the exopeptidases Kexlp and DPAP-A, have also been shown to be transported to the vacuole upon deletion of their cytoplasmic domains (Cooper and Bussey, 1992; Roberts et al., 1992). These observations, together with the finding that no particular topologic domain of the vacuolar integral membrane protein DPAP-B is necessary for targeting to the vacuole (Roberts et al., 1992), have led to the notion that transport to the vacuole is the default pathway for membrane-bound proteins in yeast cells.

Yeast cells thus stand in contrast to mammalian cells, for which there is considerable evidence that the default pathway for membrane protein transport leads to the plasma membrane. For instance, deletion of cytoplasmic ER retention signals (Pääbo et al., 1987), lysosomal targeting signals (Williams and Fukuda, 1990; Mathews et al., 1992), and even the TGN38 tail (Bos et al., 1993; Luzio et al., 1990) results in accumulation of the truncated proteins at the plasma membrane. In light of these contrasting views of default pathways in mammalian and yeast cells, the obvious similarities in the trafficking of cytoplasmically truncated furin and Kex2p are intriguing. The interpretation that is most consistent with our data, and with the body of literature on protein trafficking in mammalian cells, is that furin must have additional information within its luminal and/or transmembrane domains that directs transport of the cytoplasmically deleted protein to the endosomal/lysosomal system. This targeting information could exist in the form of a discrete structural motif or of a general physicochemical property of the protein. The existence of sorting information within topologic domains other than cytoplasmic domains has precedent in studies of the mannose 6-phosphate receptor, for which the luminal domain was shown to detain the receptor within the endosomal system (Dintzis et al., 1994).

**Concluding Remarks**

In summary, our experiments have demonstrated that two prominent features of furin trafficking within cells, TGN localization and lysosomal turnover, are mediated by information contained within different regions of the molecule. The cytoplasmic domain acts to maintain furin within the TGN, a finding that reaffirms the existence of a mechanism for TGN localization dependent upon recognition of cytoplasmic determinants. The tendency of furin to move into a lysosomal compartment, on the other hand, must be directed by a determinant encoded within the luminal and/or transmembrane domains of the molecule. Experiments are now underway to identify the nature of both types of structural determinants.

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