Interaction of Mint3 with Furin regulates the localization of Furin in the trans-Golgi network

Jinbo Han[1,2,*], Yiguo Wang[1,*], Suming Wang[3,*] and Chengwu Chi[1,2,‡]

[1]Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, People's Republic of China [2]Institute of Protein Research, Tongji University, Shanghai 200092, People's Republic of China [3]School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People's Republic of China

*These authors contributed equally to this work

‡Author for correspondence (e-mail: zwqi@sibs.ac.cn)

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Summary

Furin is a proprotein convertase that cycles between the plasma membrane, endosomes and the trans-Golgi network (TGN), maintaining a predominant distribution in the latter. Mint3, a member of the Mint protein family, is involved in the signaling and trafficking of membrane proteins. Until now, little has been known about the roles of Mint3 in the localization or trafficking of Furin. Here, using co-immunoprecipitation and immunofluorescence assays, we show that Mint3 interacts with Furin in the Golgi compartment of HeLa cells. Knockdown of endogenous Mint3 expression by RNA interference disrupts the TGN-specific localization of Furin and increases its distribution in endosomes. We further demonstrate that the phosphotyrosine-binding (PTB) domain of Mint3 is essential for the binding of Furin and that this binding affects the TGN-specific localization of Furin. Moreover, mutation studies of Furin indicate that Mint3 regulates Furin distribution mainly through interaction with the acidic peptide signal of Furin. Collectively, these data suggest that the interaction between the PTB domain of Mint3 and the acidic peptide signal of Furin regulates the specific localization of Furin in the TGN.

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Key words: Acidic peptide signal, Furin, Mint3, Phosphotyrosine-binding domain, Trans-Golgi network

Introduction

Membrane proteins are transferred between cellular organelles by using a variety of membrane-transport pathways (Creemers et al., 2001; Schapiro et al., 2004). In addition to the degradative pathways, a large group of membrane proteins are internalized and delivered to the trans-Golgi network (TGN) (Milgram et al., 1993; Molloy et al., 1994; Reaves et al., 1993; Varlamov et al., 1999). Localization and trafficking of these proteins are achieved through the recognition of short sequence motifs within the cytoplasmic domains by cellular targeting proteins, including adaptor protein (AP) complexes such as AP-1 and AP-2 (Boll et al., 1996; Hirst and Robinson, 1998; Ohno et al., 1995). Furin is a type-I membrane-associated subtilisin-like eukaryotic proprotein convertase that cycles between the plasma membrane, endosomes and the TGN, maintaining a predominant distribution in the latter. Furin proteolytically cleaves a diverse range of proprotein substrates immediately after the consensus sequence RX[K/R]R (Nakayama, 1997). Substrates of Furin include endogenous secretory proteins and viral glycoproteins of the exocytotic pathway, as well as bacterial toxins at the cell surface and in endosomes, indicating an important biological role of Furin in the activation of proproteins in multiple cellular compartments (Thomas, 2002).

The steady-state distribution of Furin in the TGN implies that slower exit to the plasma membrane is coupled with rapid internalization and retrieval to the TGN. Previous studies have established a model to explain the mechanism by which Furin cycles between the plasma membrane and the TGN. In this model, Furin trafficking includes two local recycling loops – one in the TGN and one at the cell surface and/or early endosomes (Molloy et al., 1999). Budding of Furin from the TGN is mediated by the binding of the tyrosine-based or dileucine-like hydrophobic sorting motifs to AP-1, which targets Furin to endosomes (Crump et al., 2001). PACS1 (phosphofurin acidic cluster sorting protein 1) connects the casein kinase 2 (CK2)-phosphorylated Furin acidic cluster to AP-1–clathrin to retrieve Furin back to the TGN from endosomes. At the cell surface, Furin can be tethered by the cytoskeletal protein filamin, which is also known as actin-binding protein ABP-280 (Liu et al., 1997). The dynamin- and/or clathrin-dependent internalization of cell-surface Furin is mediated by a tyrosine-based motif (YKGL) of Furin; this motif is recognized by AP-2 (Teuchert et al., 1999a). Once inside early endosomes, Furin molecules are dephosphorylated by specific protein phosphatase 2A (PP2A) isoforms and delivered to the TGN through a late endosomal compartment (Molloy et al., 1998). The dynamic cycling of Furin between the plasma membrane and the TGN is the result of many protein interactions that regulate the trafficking and localization of Furin. However, the detailed mechanism of these interactions is not completely defined.

There is emerging evidence that the Mint (also known as X11) family of adaptor proteins is involved in protein trafficking (Biederer and Sudhof, 2000). Mint proteins are composed of a long isoform-specific N-terminal sequence, a central phosphotyrosine-binding (PTB) domain and two C-terminal PSD-95/DLG-A/ZO-1 (PDZ) domains (Rogelj et al., 2006). Mint1 (also known as Apba1 and X11α) and Mint2 (also known as Apba2 and X11β) are expressed primarily in neurons, whereas Mint3 (also known as Apba3 and X11γ) is ubiquitously expressed (Okamoto and Sudhof, 1998). Mint proteins have been shown to be essential for vesicle exocytosis, and are involved in the modulation of amyloid precursor protein

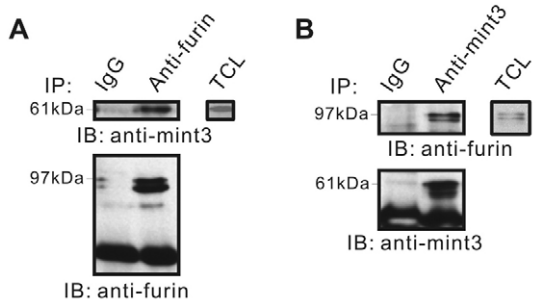

Fig. 1. Mint3 interacts with Furin in HeLa cells. The association of Mint3 with Furin in HeLa cell lysates is shown, as detected by co-immunoprecipitation (A) and reverse co-immunoprecipitation (B) assays. TCL (total cell lysate) is shown as 1% of input.

(APP) processing and the accumulation of amyloid β (Aβ) (Biederer et al., 2002; McLoughlin and Miller, 1996; Okamoto and Sudhof, 1997; Tanahashi and Tabira, 1999). Mint3 influences vesicular trafficking in various cell types and has recently been proposed to act as an Arf GTPase-dependent vesicle coat protein, thereby contributing to the trafficking of a subset of proteins to the plasma membrane (Hill et al., 2003). The role of Mint3 in the regulation of protein trafficking was confirmed on the basis of its interactions with the multi-functional cytoplasmic Bcr protein (Malmberg et al., 2004), with Rab6 GTPase (Teber et al., 2005) and with MT-MMP5 (Wang et al., 2004). Because the localization and trafficking patterns of Furin are very similar to that of MT-MMP5, we hypothesized that Mint3 was also involved in the regulation of Furin distribution. In this study, we identified Mint3 as a novel binding partner of Furin, with a functional role in regulating the localization of Furin to the TGN. We also show that the PTB domain of Mint3 preferentially binds an acidic peptide signal within the cytoplasmic domain of Furin, thereby resulting in the retention of Furin in the TGN. Our results are the first to show a role for the adapter protein Mint3 in regulating the localization and trafficking of Furin.

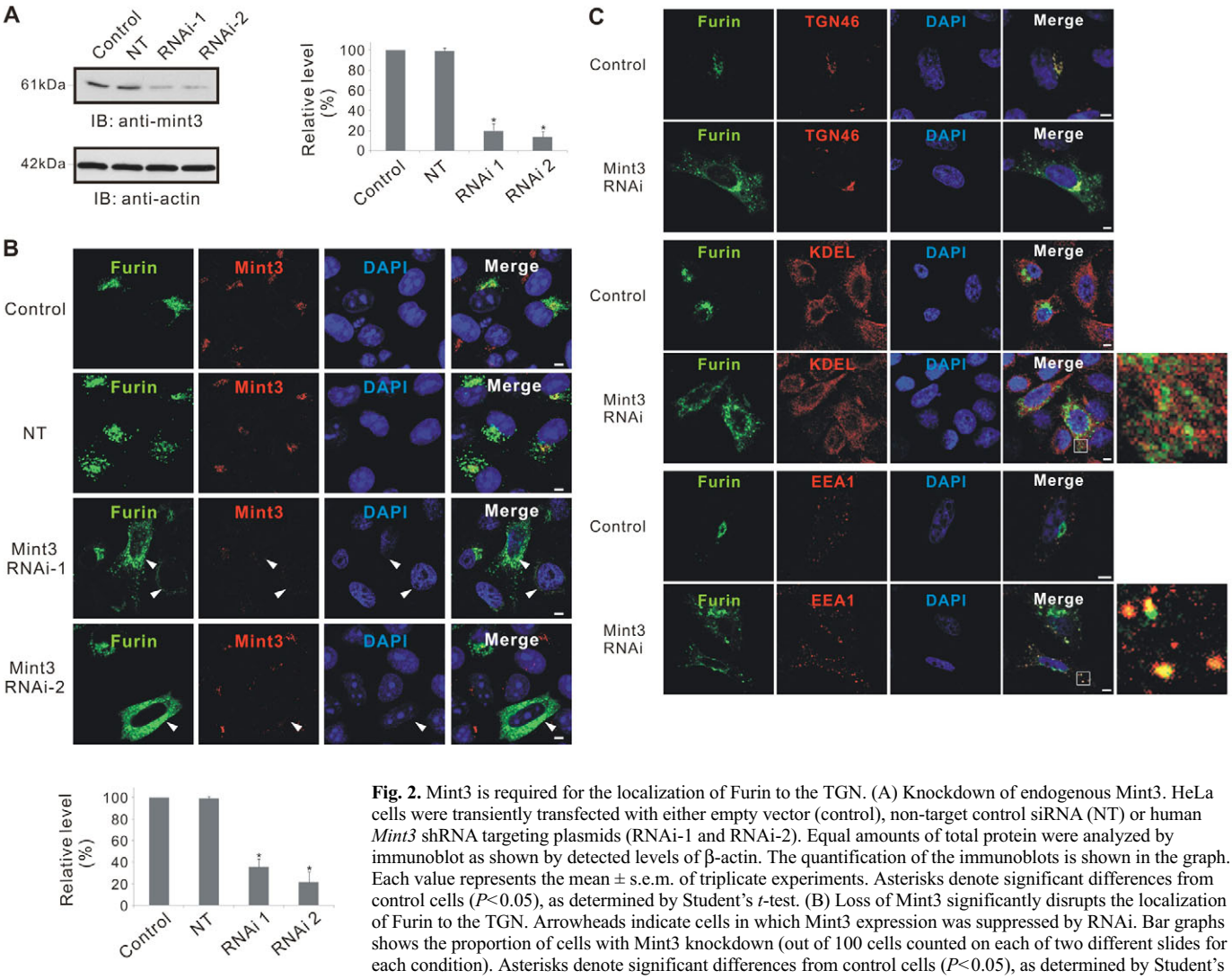

Fig. 2. Mint3 is required for the localization of Furin to the TGN. (A) Knockdown of endogenous Mint3. HeLa cells were transiently transfected with either empty vector (control), non-target control siRNA (NT) or human *Mint3* shRNA targeting plasmids (RNAi-1 and RNAi-2). Equal amounts of total protein were analyzed by immunoblot as shown by detected levels of β-actin. The quantification of the immunoblots is shown in the graph. Each value represents the mean ± s.e.m. of triplicate experiments. Asterisks denote significant differences from control cells ($P<0.05$), as determined by Student's *t*-test. (B) Loss of Mint3 significantly disrupts the localization of Furin to the TGN. Arrowheads indicate cells in which Mint3 expression was suppressed by RNAi. Bar graphs shows the proportion of cells with Mint3 knockdown (out of 100 cells counted on each of two different slides for each condition). Asterisks denote significant differences from control cells ($P<0.05$), as determined by Student's *t*-test. (C) Subcellular localization of Furin in control and Mint3-knockdown cells. HeLa cells transiently transfected with either empty vector (control) or human *Mint3* shRNA plasmid (RNAi-2) were fixed and immunostained with antibodies to Furin and either TGN46, KDEL or EEA1 as indicated. The enlarged regions of the boxed areas are shown to the right. Scale bars: 5 μm.

Results

Characterization of Furin-Mint3 interaction

Low expression levels of endogenous Furin protein are not detectable in HeLa cells by direct immunoblot or immunostaining (van Duijnhoven et al., 1992). Therefore, we transfected HeLa cells with a plasmid to express exogenous Furin under the control of a cytomegalovirus promoter (pCMV-Furin). To determine whether Mint3 can bind Furin, transfected HeLa cell lysates were immunoprecipitated with anti-Furin antibody, or with a control IgG, and immunoblotted. Mint3 was found to co-immunoprecipitate with Furin, but not with the control IgG (Fig. 1A). Similarly, reciprocal assays demonstrated that Furin could be co-immunoprecipitated with Mint3, but not with control IgG (Fig. 1B). Two bands of Furin were detected with molecular weights of approximately 96 and 90 kDa, representing the proprotein and mature forms of Furin, respectively (Leduc et al., 1992) (Fig. 1A,B).

Previous studies, including our own recent work on Furin inhibitor, demonstrated the steady-state localization of Furin in the TGN in HeLa cells (Han et al., 2007) and in BSC-40 cells (Molloy et al., 1994). Mint3 has also been reported to locate in the TGN in A431 and MDCK cells (Okamoto et al., 2001; Wang et al., 2004). To investigate the cellular localization of Furin and Mint3 in HeLa cells, we transfected Furin into HeLa cells that had endogenous Mint3 and co-stained the cells with anti-Furin and anti-Mint3 antibodies. Immunostaining demonstrated that both Mint3 and Furin have an intense perinuclear Golgi-like staining (supplementary material Fig. S1). Taken together, the data show that Mint3 interacts with Furin.

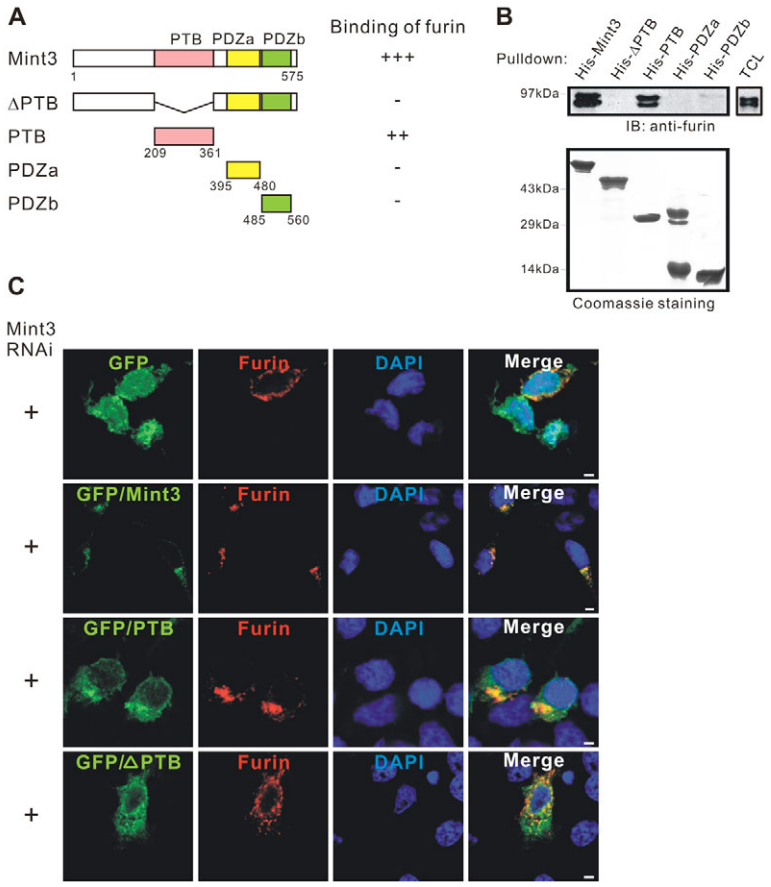

Fig. 3. The PTB domain of Mint3 is functionally required for localization of Furin in the TGN. (A) Schematic representation of Mint3 and its various deletion mutants. The binding abilities of His-tagged fusion proteins to Furin are summarized to the right based on the pull-down assays shown in B. (B) Mint3 interacts with Furin through its PTB domain. His-tagged fusion proteins were immobilized on TALON metal affinity beads and incubated with HeLa cell lysates. The upper panel shows a specific immunoblot for Furin from the pull-down lysates, and the lower panel represents the Coomassie staining of His-tagged fusion proteins in the pull-down assays. TCL (total cell lysate) is shown as 1% of input. (C) The PTB domain of Mint3 is necessary for the localization of Furin at the TGN. HeLa cells were co-transfected with pCMV-Furin, human *Mint3* shRNA plasmids and with the GFP-tagged mouse *Mint3* constructs indicated in the left panel. Cells were fixed and immunostained with antibodies to Furin and Mint3 as indicated. Scale bars: 5 μm.

Mint3 regulates the localization of Furin in the TGN

As an adaptor protein, Mint3 binds the cytoplasmic tail of membrane proteins and facilitates vesicle exocytosis (Rogelj et al., 2006). To determine whether Mint3 is specifically involved in the trafficking of Furin, we downregulated the expression of endogenous Mint3 using RNA interference (RNAi) specifically targeting *Mint3* mRNA. As shown in Fig. 2A, the expression level of endogenous Mint3 was significantly decreased to around 20% relative to the control by introducing two different *Mint3* shRNA plasmids (see Materials and Methods). To determine whether the knockdown of Mint3 expression results in abnormal localization of Furin, we performed immunostaining of HeLa cells transiently transfected with *Mint3* shRNA plasmids. Compared with control cells and cells transfected with non-target control siRNA (Fig. 2B, NT), the localization of Furin was dramatically changed from perinuclear Golgi-like staining to a diffuse pattern of fluorescence within the cytoplasm (Fig. 2B, arrowheads). These data suggest that the retention of Furin in the TGN and/or retrieval of Furin from endosomes or the plasma membrane was impaired.

To investigate the subcellular localization of Furin in Mint3-knockdown cells, we immunostained cells with Furin and with different subcellular markers. As shown in Fig. 2C, knockdown of Mint3 expression did not change the staining patterns of TGN46 (TGN marker), KDEL [endoplasmic reticulum (ER) marker] or EEA1 (endosome marker) compared with the control panels, indicating the integrity of the subcellular compartments. To further exclude the possibility that changes in Furin distribution were the result of a compromised Golgi compartment in Mint3-knockdown cells, we used another marker protein of the Golgi complex, GM130, to check the integrity of the Golgi complex. As shown in supplementary material Fig. S2, the distribution of GM130 in Mint3-RNAi cells was similar to that observed in control cells, suggesting that the abnormal localization of Furin in Mint3-RNAi cells was a result of downregulated Mint3 expression. The diffused distribution pattern of Furin staining in Mint3-RNAi cells was quite different from that of TGN46 and KDEL staining. By contrast, most of the diffused Furin colocalized well with EEA1 (Fig. 2C). These results suggest that more Furin molecules localized to endosome when Mint3 expression was knocked down. Therefore, we speculate that more Furin molecules are at the cell surface of Mint3-knockdown cells. In order to confirm this speculation, we carried out in vitro Furin activity assay (Liu et al., 2004). As shown in supplementary material Fig. S3, compared

with control cells, knockdown of Mint3 expression increased Furin activity at the cell surface. In short, knockdown of Mint3 decreases the retention of Furin at the TGN and increases the distribution of Furin both at endosomes and on membrane.

The PTB domain of Mint3 is essential for Furin-Mint3 interaction

Mint3 is composed of one central PTB domain and two C-terminal PDZ domains that have been characterized for their ability to bind various membrane proteins (Okamoto and Sudhof, 1998). To identify the specific region of Mint3 that interacts with Furin, we expressed and purified full-length Mint3, a deletion-mutant Mint3 without the PTB domain (ΔPTB), as well as individual domains of Mint3 – PTB, PDZa and PDZb – as His-tagged fusion proteins (Fig. 3A). Pull-down assays showed that Furin could bind both the full-length Mint3 and its individual PTB domain; however, it could not bind ΔPTB and either of the two PDZ domains (Fig. 3A,B), suggesting that Furin binds to the PTB domain of Mint3.

To investigate the biological significance of the Mint3 PTB domain in the binding of Furin and the regulation of Furin localization in the TGN, we transiently co-transfected various GFP-tagged mouse-derived Mint3 constructs with *Mint3* shRNA plasmids. At first, to investigate the rescue effect of different mouse-derived Mint3 plasmids, the specificity of Mint3 short hairpin RNA (shRNA) was confirmed, which was specific to human-derived *Mint3* but not to mouse-derived *Mint3* (data not shown). Both full-length Mint3 (GFP-Mint3) and its PTB domain (GFP-PTB) were localized to the TGN in immunofluorescence studies, although the ability of GFP-PTB to localize to the TGN was weaker than GFP-Mint3 (Fig. 3C). By contrast, the Mint3 deletion mutant lacking the PTB domain (GFP-ΔPTB) was observed to be diffusely distributed in the cytoplasm. In addition, overexpression of both GFP-Mint3 and GFP-PTB rescued the TGN-specific distribution of Furin in Mint3-RNAi cells, whereas overexpression of GFP-ΔPTB and GFP alone did not. Taken together, these data suggest that the PTB domain of Mint3 is an essential element in regulating the localization of Furin to the TGN.

Mint3 mainly binds the acidic peptide signal in the cytoplasmic domain of Furin

Furin contains: a signal peptide that directs translocation of the pro-enzyme into the ER; a prodomain that has a crucial role in the folding, activation and transport of Furin; a conserved catalytic domain (CA) with the aspartate (Asp), histidine (His) and serine (Ser) residues that form the catalytic triad; a P domain (P), which is essential for enzyme activity; and a cytoplasmic domain (CP) that controls the localization and sorting of Furin in the TGN-endosomal system (Nakayama, 1997). To investigate which domain of Furin is essential for the binding of Mint3 to Furin, the catalytic domain (CA), P domain (P) and cytoplasmic domain (CP) of Furin were fused to GST and purified for pull-down assays (Fig. 4A). The proteins from HeLa cell lysates that associated with the fusion proteins in pull-down assays were detected by immunoblot (Fig. 4B). As indicated in Fig. 4B, only GST-CP bound Mint3 (Fig. 4A,B), indicating that interactions between Furin and Mint3 are mediated by the cytoplasmic domain of Furin, and, based on the assays in Fig. 3, the PTB domain of Mint3.

Previous work has identified two independent targeting signals in the cytoplasmic domain of Furin that determine its localization to TGN and its endosomal trafficking (Schafer et al., 1995).

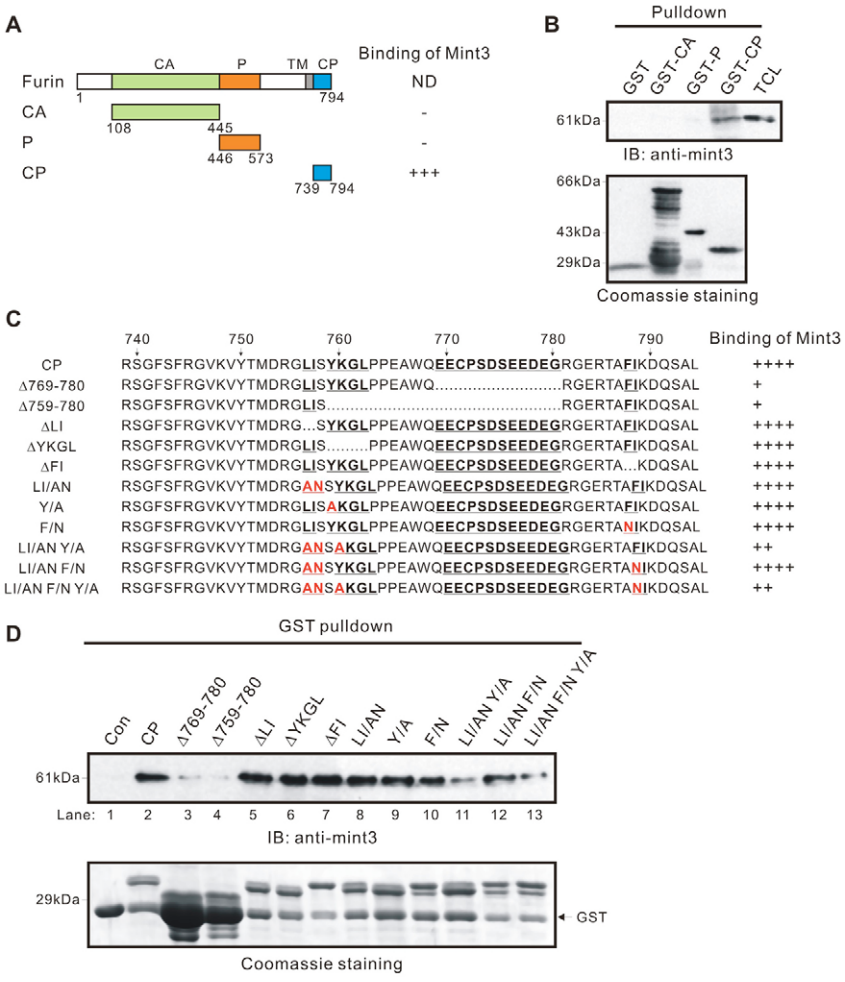

Fig. 4. Binding of the acidic peptide region of Furin by Mint3. (A) Schematic diagrams of Furin and its various deletion mutants. The binding abilities of GST-tagged fusion proteins to Mint3 are summarized to the right. ND, not determined. (B) The association of the cytoplasmic domain of Furin with Mint3. GST fusion proteins of the catalytic domain (GST-CA), the P-domain (GST-P) and the cytoplasmic domain (GST-CP) of Furin were immobilized on glutathione-Sepharose beads and incubated with lysates from HeLa cells. Bound proteins were eluted, separated by SDS-PAGE and visualized as indicated. TCL (total cell lysate) is shown as 5% of input. (C) Schematic representation of GST fusion proteins with various mutations of the Furin cytoplasmic domain. Deleted regions are marked by a dotted line and amino acid substitutions are labeled in red. Binding motifs are bold and underlined. The binding abilities of GST-tagged fusion proteins to Mint3 are summarized to the right. (D) The mutants with acidic peptide deletion (Δ769-780) fail to bind Mint3 efficiently, and the mutants with LI/AN Y/A substitutions decrease the binding affinity between Furin and Mint3.

These two signal sequences consist of an acidic peptide: $CPSDSEEDEG_{780}$, and three hydrophobic motifs: a tyrosine ($YKGL_{762}$) motif, a leucine-isoleucine (LI_{757}) motif and a phenylalanine (Phe_{787}) motif (Schafer et al., 1995; Teuchert et al., 1999a). To investigate which element of Furin is involved in the binding of Furin to Mint3, we constructed various Furin mutants as GST-fusion proteins (Fig. 4C). Our binding studies (Fig. 4C,D and supplementary material Fig. S4) showed that endogenous Mint3 or recombinant His-Mint3 bound various GST-Furin mutants (ΔLI, ΔYKGL, ΔFI, LI/AN, Y/A, F/N, LI/AN F/N; see Fig. 4C for details) as efficiently as wild-type GST-CP. By contrast, the Furin mutants Δ769-780 (with a deleted acidic cluster), and Δ759-780 (lacking both the YKGL motif and the acidic cluster) showed low-affinity interactions with Mint3, suggesting that the acidic cluster region of Furin is an essential binding site of Mint3. In addition, the binding efficiency of the Furin mutants (LI/AN Y/A) and (LI/AN F/N Y/A) to Mint3 decreased (Fig. 4D and supplementary material Fig. S4, lanes 11 and 13), suggesting that the hydrophobic motifs (LI and YKGL) might be the minor binding sites of Mint3.

Discussion

Furin has important roles in embryogenesis, homeostasis and various diseases, including Alzheimer disease (Thomas, 2002). Therefore, it is important to study the mechanisms involved in the localization and trafficking of Furin. Similar to other type-I membrane proteins, the dynamic cycling of Furin between the plasma membrane and TGN is controlled by the binding of the sorting motifs of Furin by other proteins (Schafer et al., 1995; Teuchert et al., 1999a; Teuchert et al., 1999b). Our data indicate that the PTB-domain-containing adaptor protein Mint3 regulates TGN localization of Furin through interactions with the cytoplasmic domain of Furin. The Mint family of adaptor proteins are well-characterized for their roles in the formation of multi-protein complexes and their ability to regulate the signaling and trafficking of membrane proteins (Rogelj et al., 2006). However, a role for Mint3 in the regulation of Furin trafficking has not previously been reported.

Analysis of the expression patterns of Mint3 and Furin has shown that the two proteins are ubiquitously expressed, with the highest expression levels in the liver (Nakayama, 1997; Okamoto and Sudhof, 1998). Furin and Mint3 also share overlapping cellular-localization patterns. Furin is concentrated in the TGN (Bosshart et al., 1994), and Mint3 is also located in the TGN and at the plasma membrane (Okamoto et al., 2001). Using RNAi knockdown, the loss of Mint3 expression resulted in a change in Furin localization from perinuclear Golgi-like staining to a diffuse distribution pattern (Fig. 2B). Subcellular distribution analysis showed that more Furin molecules localized to endosomes in Mint3-RNAi cells (Fig. 2C), suggesting a role of Mint3 in the retention of Furin in the TGN.

Mint3 has been reported to bind to a wide variety of proteins via its PTB and PDZ domains (Teber et al., 2005). For example, binding between Rab6A and APP mediated by Mint3 has been shown to require the complete PTB domain of Mint3. These findings suggest that the PTB domain of Mint3 is important in mediating protein-protein interactions. Studies by Okamoto et al. showed that the PTB and PDZb domains are both responsible for the localization of Mint3 in the TGN (Okamoto et al., 2001). Our work here also shows that the PTB domain of Mint3 was required for the binding of Furin, and was associated with providing TGN-specific localization of Furin (Fig. 3). Thus, the inability of the Mint3 mutant GFP-ΔPTB to restore Furin localization to the TGN in Mint3-RNAi cells (Fig. 3C) might be caused either by failure of Mint3 to localize to the TGN, or by failure of Mint3 to interact with Furin owing to deletion of its PTB domain.

TGN localization of Furin and its dynamic cycling are controlled by sequences within its C-terminal 56 amino acids. Two independent targeting signals – the acidic peptide signal $CPSDSEEDEG_{780}$ and a combination of three hydrophobic motifs (YKGL, FI and LI) – control the local cycling of Furin between the TGN and endosome (Teuchert et al., 1999a). Binding of the hydrophobic segment to the adaptor protein AP-1 is required for the budding of Furin from the TGN to endosome, whereas binding of the CK2-phosphorylated Furin acidic cluster to the sorting protein PACS1 is involved in the efficient retrieval of endosomal Furin to the TGN (Scott et al., 2003). However, recent studies found that PACS1 knockdown has no effect on Nef-induced downregulation of HLA-A2 nor on the steady-state distribution of Furin in HeLa cells (Lubben et al., 2007). Thus, PACS1 might play some sorting roles at endosomes relating to the phosphorylation state of Furin, whereas Mint3 might play major roles in the retention of Furin in the TGN. Our results show that Mint3 can bind mainly to the acidic peptide and to some extent to the hydrophobic motifs of Furin via its PTB domain (Fig. 4). It is possible that Mint3 retains most of the Furin molecules in the TGN by binding to the acidic cluster. By contrast, Mint3 might compete

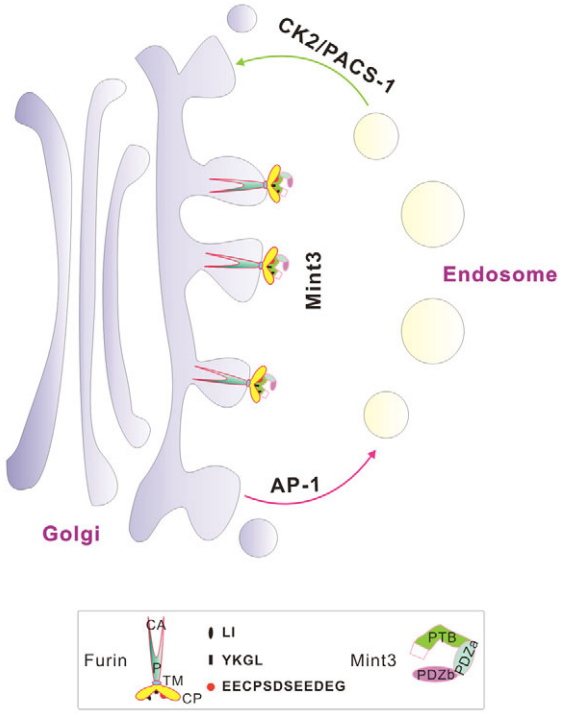

Fig. 5. A model of the regulation of Furin TGN localization by Mint3. Binding of the hydrophobic motif to the adaptor protein AP-1 is required for the budding of Furin from the TGN to endosomes, whereas binding of CK2-phosphorylated Furin acidic cluster to the sorting protein PACS1 transports Furin back from endosomes to the TGN (Thomas, 2002). In the TGN, Mint3 mainly binds to the acidic cluster of Furin and retains most of the Furin molecule in this compartment. However, binding of the YKGL and LI motifs of Furin with Mint3 might compete its binding with AP-1 and hence reduce the budding of Furin from the TGN. CA, catalytic domain; CP, cytoplasmic domain; P, P domain; TM, transmembrane domain.

with the binding Furin to AP-1 in YKGL and LI sites, and hence reduces the budding of Furin from the TGN (Fig. 5). Tethering of Furin in the TGN by Mint3 might also serve as a strategically located reservoir of Furin molecules that are not temporarily required for proprotein processing. The steady-state distribution of Furin in the TGN is the final output determined by the balance between retention and dynamic trafficking. In summary, our results show that Mint3 interacts with Furin and regulates the TGN localization of this protein; this aids not only our understanding of the localization and dynamic trafficking of Furin but also the further study of the physiological and pathological roles of Furin.

Materials and Methods

Plasmids and reagents

The full-length *Mint3* gene from human and mouse were obtained by reverse transcriptase (RT)-PCR from HeLa cells and mouse brain, respectively. Full-length human *Mint3*, and its PTB, PDZa and PDZb domains, were cloned to pET28a. The primers used are as follows: Mint3, 5′-GCGGAATTCATGGACTTCCCCACAATTTCC-3′ (*Eco*RI), 5′-GCGAGCGGCCGCCAGGTACACGGGCTGCTCCTG-3′ (*Not*I); PTB, 5′-GCGGAATTCTGTGACCATGAAGACCTCCTG-3′ (*Eco*RI), 5′-GCGAGCGGCCGCACCGCTTTCCCGTAGGAAC-3′ (*Not*I); PDZa, 5′-GCGGAATTCGTGCACCTCGAGAAGCGGCGA-3′ (*Eco*RI), 5′-GCGAGCGGCCGCGCAGTGGACGATGCTGAGTGT-3′ (*Not*I); PDZb, 5′-GCGGAATTCACCGCCATCATCCACCGGCCC-3′ (*Eco*RI), 5′-GCGAGCGGCCGCAGCTGGCATCGTCTTGATATG-3′ (*Not*I). The full-length mouse *Mint3* gene was obtained by RT-PCR from mouse brain and ligated to pEGFP for expression of GFP-fusion protein. The PTB domain and ΔPTB of mouse *Mint3* were also cloned to pEGFP using the same system. The primers used are as follows: GFP/mMint3, forward, 5′-CGGAATTCTATGGAGTTCCTGCCAGGACC-3′ (*Eco*RI), reverse, 5′-CGGGATCCTAGGTATACTGGTTGTTCCTGTC-3′ (*Bam*HI); GFP/PTB, forward, 5′-CGGAATTCTGATGGTGTCATCTTCGGGGC-3′ (*Eco*RI), reverse, 5′-CGGGATCCGATCCTGTTCTCCTGTAGGAA-3′ (*Bam*HI); GFP/ PTB, forward half, 5′-ACGCGTCGACCATGAGCTCCTCCGGCCCAC-3′ (*Sal*I), backward half, 5′-ACGCGTCGACGACCCCAGCCAGGTGGGCA-3′ (*Sal*I).

The plasmid pCMV-Furin was a kind gift from G. Thomas (The Oregon Health Sciences University, Portland, OR). The catalytic domain (CA), P domain (P) and cytoplasmic domain (CY) of Furin were subcloned from full-length pCMV-Furin and ligated in-frame to the C-terminus of GST using the PGEX-6P system (Pharmacia). The primers used are as follows: CA, 5′-GCGAATTCGACGTGTACCAGGAGCCCACA-3′ (*Eco*RI), 5′-GCGAGCGGCCGCGGCCACTGTGGTCCAATTCTG-3′ (*Not*I); P, 5′-GCGAATTCCCCCAGCGGAAGTGCATCATC-3′ (*Eco*RI), 5′-GCGAGCGGCCGCGGTGCCATAGAGTACGAGGGT-3′ (*Not*I); CY, 5′-GCGAATTCCGCTCTGGCTTTAGTTTTCGG-3′ (*Eco*RI), 5′-GCGAGCGGCCGCGAGGGCGCTCTGGTCTTTGAT-3′ (*Not*I).

Primers used for Furin mutants with deletions and substitutions of amino acids in the cytoplasmic tail are as follows: Δ759-780, 5′-CGGGGCGAGAGGACCGCCTTT-3′, 5′-GGAGATGAGGCCACGGTCCAT-3′; Δ769-780, 5′-CGGGGCGAGAGGACCGCCTTTATC-3′, 5′-CTGCCAGGCTTCAGGGGGCAGCCC-3′; ΔLI, 5′-TCCTACAAGGGGCTGCCCCCTGAA-3′, 5′-GCCACGGTCCATGGTGTACACCTT-3′; ΔYKGL, 5′-CCCCCTGAAGCCTGGCAGGAGGAG-3′, 5′-GGAGATGAGGCCACGGTCCATGGT-3′; ΔFI, 5′-AAAGACCAGAGCGCCCTC-3′, 5′-GGCGGTCCTCTCGCCCCGGCCCTC-3′; LI/AN, 5′-GCCAACTCCTACAAGGGGCTGCCCCCT-3′, 5′-GCCACGGTCCATGGTGTACAC-3′; Y/A, 5′-GCCAAGGGGCTGCCCCCTGAAGCC-3′, 5′-GGAGATGAGGCCACGGTCCAT-3′; F/N, 5′-AACATCAAAGACCAGAGCGCCCTC-3′, 5′-GGCGGTCCTCTCGCCCCGGCC-3′; LI/AN + Y/A, 5′-GCCAACTCCGCCAAGGGGCTGCCCCCTGAAGCC-3′, 5′-GCCACGGTCCATGGTGTACAC-3′.

Vector-based shRNAs of human *Mint3* were cloned into pSilencer 3.1 (Ambion) using *Bam*HI and *Hin*dIII sites. The sequences of the human-derived *Mint3* oligonucleotides used are: RNAi-1 shRNA sense primer, 5′-GATCCGATGCTCTGCCACGTATTCTTCAAGAGAGAATACGTGGCAGAGCATCTTTTTTGGAAA-3′; RNAi-1 shRNA anti-sense primer, 5′-AGCTTTTCCAAAAAAGATGCTCTGCCACGTATTCTCTCTTGAAGAATACGTGGCAGAGCATCG-3′; RNAi-2 shRNA sense primer, 5′-GATCCGAGGATCAAGGTCTTGACATTCAAGAGATGTCAAGACCTTGATCCTCTTTTTTGGAAA-3′; RNAi-2 shRNA anti-sense primer, 5′-AGCTTTTCCAAAAAAGAGGATCAAGGTCTTGACATCTCTTGAATGTCAAGACCTTGATCCTCG-3′. Non-target control siRNA (Silencer negative control #1 siRNA) was from Ambion.

The anti-Furin antibody was purchased from Alexis. The anti-Mint3 antibody was from BD Pharmingen. The anti-β-actin antibody was purchased from Sigma. The anti-TGN46, anti-KDEL and anti-EEA1 antibodies were from Abcam. Secondary antibodies for the immunoblot and immunofluorescence assays were purchased from Sigma and Molecular Probes, respectively. The fluorogenic substrate pyrArg-Thr-Lys-Arg-7-amino-4-methylcoumarin (MCA) was purchased from Bachem Bioscience. All general chemicals were purchased from Sigma-Aldrich or Fluka.

Cell culture and transfection

HeLa and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. The cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation

At 48 hours after transfection with pCMV-Furin, HeLa cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, a cocktail of protease inhibitors purchased from Roche) and cleared by centrifugation. The supernatant was incubated at 4°C with anti-Furin (1 μg) or anti-Mint3 (1 μg) antibodies overnight. Immune complexes were immobilized on protein-G beads for 3 hours, washed thrice with the lysis buffer and denatured in SDS sample buffer at 100°C for 10 minutes. The prepared samples were subjected to immunoblot analysis.

Pull-down assays

GST or His-tag fusion proteins were expressed in *Escherichia coli* and purified according to the manufacturer's instructions. For GST pull-down assays, HeLa cell lysates were centrifuged and the supernatants were incubated with 1 μg GST or GST fusion proteins overnight. Glutathione-Sepharose beads were added and incubated for an additional 3 hours. The beads were washed and boiled in SDS sample buffer. For His-tag pull-down assays, HeLa cells transfected with pCMV-Furin were lysed and the cell extracts were cleared and then incubated with 1 μg His-tag fusion protein overnight. TALON metal affinity beads (Clontech) were added and incubated for an additional 3 hours. The beads were washed and boiled in SDS sample buffer. The prepared samples were analyzed by Coomassie staining or immunoblot as shown in the Results section.

Immunostaining and confocal microscopy

Cells were grown on glass coverslips (Fisher Scientific) and co-transfected with pCMV-Furin and pSilencer 3.1 plasmids (control) or with pCMV-Furin and human *Mint3* shRNA targeting plasmids (RNAi-1 and RNAi-2). After 48 hours, cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes. After incubation with anti-Furin (1:300) and anti-Mint3 (1:250) antibodies, slides were rinsed in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) and incubated with the corresponding secondary antibodies. Images were captured using a CCD camera (model DC350F; Leica) on a confocal microscope (model DM5000B; Leica).

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