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Research article

SYNTAXIN 8 HAS TWO FUNCTIONALLY DISTINCT DI-LEUCINE-BASED MOTIFS

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Abstract: Syntaxin 8 has been shown to form the SNARE complex with syntaxin 7, vti1b and endobrevin. These have been shown to function as the machinery for the homotypic fusion of late endosomes. Recently, we showed that syntaxins 7 and 8 cycle through the plasma membrane, and that the di-leucine-based motifs in the cytoplasmic domain of syntaxins 7 and 8 respectively function in their endocytic and exocytic processes. However, we could not elucidate the mechanism by which syntaxin 8 cycles through the plasma membrane. In this study, we constructed several different syntaxin 8 molecules by mutating putative di-leucine-based motifs, and analyzed their intracellular localization and trafficking. We found a di-leucine-based motif in the cytoplasmic domain of syntaxin 8. It is similar to that of syntaxin 7, and functions in its endocytosis. These results suggest that in the cytoplasmic domain, syntaxin 8 has two functionally distinct di-leucine-based motifs that act independently in its endocytic and exocytic processes. This is the first report on two di-leucine-based motifs in the same molecule acting independently in distinct transport pathways.

Key words: Syntaxin, Di-leucine-based motif, Endocytosis, Exocytosis

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Abbreviations used: HA – hemagglutinin; NSF – N-ethylmaleimide-sensitive factor; SNAP – soluble NSF-attachment protein; SNARE – SNAP-receptor; TGN – *trans*-Golgi network; t-SNARE – target-SNARE; v-SNARE – vesicle-SNARE; vti1b – Vps10p tail interactor 1b

INTRODUCTION

In eukaryotic cells, exocytic and endocytic protein transport is mediated by various types of transport vesicles that move between intracellular compartments. These transport vesicles bud from donor membrane compartments and fuse with target membrane compartments [1-5]. N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs) participate in many different transport events [6-8]. SNAP receptors (SNAREs) are thought to coordinate the actions of NSF and SNAPs, thus directing the process of intracellular vesicle fusion [3]. This hypothesis predicts that the docking of transport vesicles to target membrane compartments is mediated by the specific pairing of v-SNAREs (members of the VAMP or synaptobrevin family found on transport vesicles) with t-SNAREs (members of the syntaxin and SNAP-25 families found on target membrane compartments) [9-14]. The presence of many isoforms of t- and v-SNAREs that uniquely localize to distinct membrane compartments may provide a basis for the specificity of the docking step in different transport pathways [9]. Due to the importance of t- and v-SNAREs in the docking and fusion of transport vesicles, the roles of SNAREs in intracellular localization must be established to understand the mechanism of transport specificity.

The efficient sorting of membrane proteins to a variety of post-Golgi destinations is controlled by sorting motifs, which are short specific sequences in the protein cytoplasmic domain [15, 16]. Two major groups of sorting signals have been identified. The first group has tyrosine-based motifs, which usually conform to the consensus YXX∅ (where X is any amino acid, and ∅ is a strong hydrophobic amino acid) or FXNPXY. The second group has di-leucine/di-hydrophobic motifs, in which one of the leucines can be replaced by isoleucine, valine, or methionine without loss of function [17-19]. Many endosome/lysosome-targeting proteins that contain di-leucine-based motif(s) in their cytoplasmic domains have been identified. Of these proteins, tyrosinase [20], low-density lipoprotein receptor-related protein [21], and CD3-γ, δ chain [17] contain both tyrosine-based and di-leucine-based motifs. These motifs are also involved in targeting proteins to their destinations and in recycling proteins from the plasma membrane. The mannose 6-phosphate/insulin-like growth factor-II receptor also contains the two motifs. The di-leucine-based motif mediates direct transport to the late endosome, while the tyrosine-based motif directs transport from the plasma membrane [22]. The MHC-associated invariant chain contains two distinct di-leucine-based motifs. These motifs contribute equally and independently both to targeting proteins to larger vesicular structures and to recycling proteins from the plasma membrane [18, 19].

Syntaxins 7 and 8, members of the t-SNARE protein family, are localized in the post-Golgi endosomal population [23-26]. Syntaxins 7 and 8 have been shown to form the SNARE complex with the t-SNARE vti1b, and the v-SNARE endobrevin, and have been shown to function as the machinery for the

homotypic fusion of late endosomes [27]. We recently demonstrated that the cytoplasmic domains of syntaxins 7 and 8 are important for intracellular localization to the endosomal populations and for internalization from the plasma membrane. We further demonstrated that the di-leucine-based motifs of syntaxins 7 and 8 respectively function as signal motifs in their endocytosis and exocytosis [28]. However, we could not elucidate the mechanism that controls the recycling of syntaxin 8 from the plasma membrane, although the cytoplasmic domain of syntaxin 8 obviously participates in internalization. Therefore, in this study, to identify the polypeptide domain that directs the recycling of syntaxin 8 from the plasma membrane, we used additional syntaxin 8 mutants, each of which was mutated in one putative di-leucine-based motif in the cytoplasmic domain.

MATERIALS AND METHODS

Antibodies

Monoclonal rat anti-hemagglutinin (HA) antibody (3F10) and monoclonal mouse anti-c-myc antibody (9E10) were purchased from Roche Diagnostics Corp. Monoclonal anti-cathepsin D antibody was purchased from BD Transduction Laboratories. All the fluorescent secondary antibodies were obtained from Jackson Immunoresearch Laboratories.

cDNA cloning and plasmid constructions

A cDNA encoding the full length of human syntaxin 8 was obtained by PCR of human liver and kidney cDNA libraries (Life Technologies, Inc). QuikChange™ Site-Directed Mutagenesis (STRATAGENE) was used to generate mutations in the putative di-leucine-based motifs found at amino acids 52-58 (M1), 77-83 (M2), 156-162 (M3), 177-183 (M4), and 184-190 (M5) of syntaxin 8. The M1 mutation of syntaxin 8 contained Leu57-Leu58 → Ala-Ala substitutions. Similarly, M2, M3, M4, and M5 respectively contained Leu82-Leu83 → Ala-Ala, Ile161-Ile162 → Ala-Ala, Ile182-Ile183 → Ala-Ala, and Leu189-Val190 → Ala-Ala substitutions. For the intracellular localization analysis, wild-type and syntaxin 8 mutant cDNAs were subcloned into the pcDNA3-HAN vector [29] for expression in mammalian cells as fusion proteins with the NH_2-termini fused to an HA tag. For antibody uptake experiments, wild-type and syntaxin 8 mutant cDNAs were subcloned into the pcDNA3-myc3C vector for expression as fusion proteins with the COOH-termini fused to three c-myc tags [28].

Cell culture and indirect immunofluorescence analysis

Rat Clone 9 hepatocytes were cultured at 37ºC in 5% CO_2 in nutrient mixture F12 medium supplemented with 10% fetal calf serum and containing 100 IU/ml penicillin and 100 µg/ml streptomycin. Clone 9 cells were grown on 8-well Lab-Tek II chamber slides and were transfected using FuGene6™ transfection reagent (Roche Diagnostics Corp). Cells were incubated for 12 and 24 hours for

intracellular localization analysis, and for 24 hours for antibody uptake experiments.

The cells were processed for indirect immunofluorescence analysis, as described previously [30]. Briefly, cells were fixed and permeabilized with methanol at -20ºC for 5 minutes. For antibody uptake experiments, the cells were incubated with 8 µg/ml monoclonal mouse anti-c-myc antibody 9E10 for 3 hours before fixing. To detect HA-tagged syntaxins, the fixed and permeabilized cells were incubated with monoclonal rat anti-HA antibody 3F10 and subsequently incubated with the secondary antibody. To detect c-myc-tagged syntaxins, fixed and permeabilized cells were incubated with FITC-conjugated anti-mouse IgG. For double-labeled intracellular localization analysis, cells were fixed with 4% paraformaldehyde and permeabilized with 20 µg/ml digitonin. The fixed and permeabilized cells were incubated with 3F10 and anti-cathepsin D antibodies, and were subsequently incubated with secondary antibodies. The stained cells were observed with a confocal laser-scanning microscope (LSM 410 invert; Carl Zeiss).

RESULTS AND DISCUSSION

Syntaxin 8 internalizes from the plasma membrane by its di-leucine-based motif

In order to identify the polypeptide domain responsible for the syntaxin 8 internalization signal, we first generated several syntaxin 8 mutants and analyzed their intracellular localization. Fig. 1 shows the amino acid sequence of human

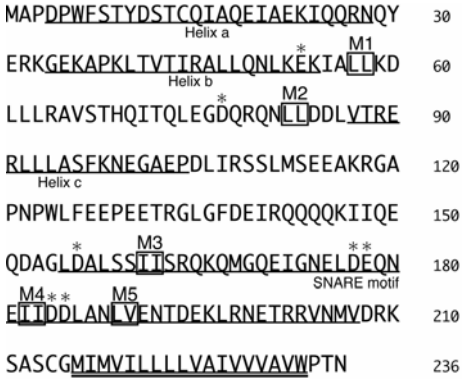

Fig. 1. Five putative di-leucine-based motifs are present in the cytoplasmic domain of syntaxin 8. Human syntaxin 8 (syn8) is composed of 236 amino acid residues. The cytoplasmic domain contains five putative di-leucine-based motifs. These motifs contain di-leucine or di-hydrophobic residues (M1-M5) and acidic residue(s) (*) positioned 4 or 5 residues NH_2-terminal to the di-leucine/di-hydrophobic residues. The putative di-leucine-based motifs were mutated as described in the Materials and Methods section. The N-terminal helical domains (helix a-c) and SNARE motif are underlined. The transmembrane domain is double-underlined.

syntaxin 8 [26, 31, 32]. There are five putative di-leucine-based motifs in the cytoplasmic domain of syntaxin 8. These motifs contain acidic residue(s) (*) positioned 4 or 5 residues NH_2-terminal to the di-leucine/di-hydrophobic residues (M1-M5). However, there is no putative tyrosine-based motif in the syntaxin 8 cytoplasmic domain. Therefore, we generated mutants with double-alanines at amino acids 57-58 (M1), 82-83 (M2), 161-162 (M3), 182-183 (M4), or 189-190 (M5) of the di-leucine/di-hydrophobic residues, respectively resulting in syn8-M1, syn8-M2, syn8-M3, syn8-M4, or syn8-M5.

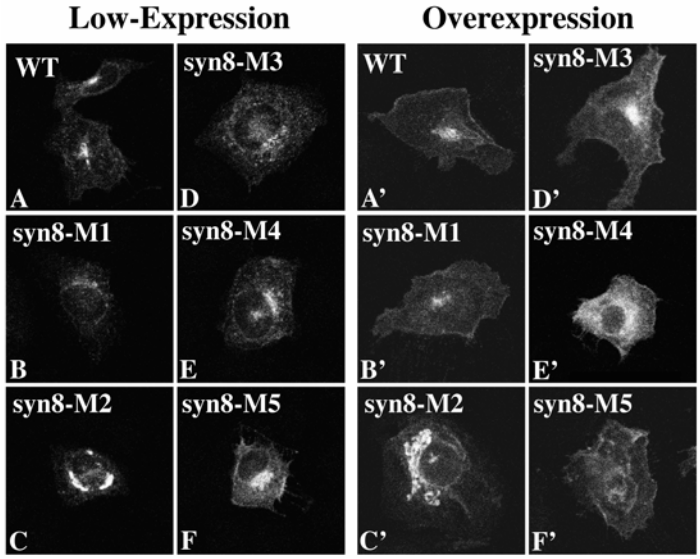

Fig. 2. Immunofluorescence analysis of di-leucine-based motif mutants. Clone 9 cells were transfected with HA-tagged wild-type (WT) or di-leucine-based motif mutants of syntaxin 8 (syn8-M1 to M5). After incubation for 12 hours (low-expression) or 24 hours (overexpression), the cells were fixed and stained with anti-HA antibody. Syn8-M2 was localized only to the perinuclear Golgi region in both low- and overexpressing cells. Syn8-M5 was efficiently localized at the plasma membrane in addition to the intracellular populations even in low-expressing cells. It was localized mainly at the plasma membrane in overexpressing cells.

It has been shown that wild-type syntaxin 8 is mainly localized to endosomal/lysosomal populations and is minimally localized at the plasma membrane [25, 26, 28]. We recently demonstrated that syn8-M2 has an exocytosis defect, and is localized at the Golgi region, even in overexpressing cells, but is not found at the plasma membrane [28]. Similar results were obtained in this study (Fig. 2C, C'). We analyzed the intracellular localization of other mutants in both low-expressing and overexpressing cells. Like wild-type syntaxin 8, syn8-M1, syn8-M3, and syn8-M4 were mainly localized in intracellular compartments in low-expressing cells (Fig. 2A, B, D, E). In

addition to the intracellular staining, some cells were slightly stained at the plasma membrane (data not shown). In overexpressing cells, these mutants, along with wild-type syntaxin 8, were mainly localized at the plasma membrane (Fig. 2A', B', D', E'). These results suggest that these putative di-leucine-based motifs have a minor role in the internalization of syntaxin 8. Syn8-M5 was more effectively localized at the plasma membrane than the wild-type and other syntaxin 8 mutants in low-expressing cells (Fig. 2F). In overexpressing cells, most syn8-M5 was localized at the plasma membrane and only a small amount was found in the intracellular populations (Fig. 2F'). These findings suggest that the M5 mutation has an endocytosis defect, and that the putative di-leucine-based motif at amino acids 184-190 of syntaxin 8 has an important role for internalization from the plasma membrane. Di-leucine-based motifs have been found to bind to the clathrin adaptor complexes AP-1, AP-2 [33], and AP-3 [34, 35]. Both AP-1 and AP-3 adaptor complexes mediate the formation of clathrin-coated vesicles at the *trans*-Golgi network (TGN), and are involved in protein transport from the TGN to the endosomal/lysosomal populations. The AP-1 adaptor complex has also been shown to be involved in protein transport from the TGN to the plasma membrane. By contrast, the AP-2 clathrin adaptor complex mediates the formation of clathrin-coated vesicles at the plasma membrane and is involved in endocytosis [36]. In Fig. 2, we showed that one syntaxin 8 di-leucine-based motif (M5; amino acids 184-190) is involved in endocytosis. Therefore, it may be possible that this di-leucine-based motif binds to the AP-2 clathrin adaptor complex, and that syntaxin 8 internalizes from the plasma membrane via a clathrin-mediated pathway.

Syntaxin 8 is directly delivered from the TGN to endosomal/lysosomal populations

Next, we performed antibody uptake experiments to clarify the endocytosis defect of the syntaxin 8 M5 mutation. Since syntaxin 8 does not contain a large enough luminal domain for antibody binding, we transiently expressed wild-type syntaxin 8 and mutants in which the COOH-termini were fused to three c-myc tags. When these tagged proteins are inserted into a membrane, the c-myc tags are positioned within the lumen of the appropriate compartment [28, 37]. Thus, the c-myc tags are exposed on the extracellular surface of cells after exocytosis, and may interact with the anti-c-myc antibody in the medium. Antibody uptake was dependent on protein cycling through the plasma membrane since cells expressing syn8-M2, which has an exocytosis defect and is not localized at the plasma membrane, did not bind the anti-c-myc antibody (Fig. 3C). Incubation of the anti-c-myc antibody with cells expressing syn8-M1, syn8-M3, syn8-M4, or wild-type syntaxin 8 resulted in efficient labeling of intracellular compartments (Fig. 3A, B, D, E). This finding indicates that the di-leucine/di-hydrophobic residues at amino acids 57-58 (M1), 161-162 (M3), and 182-183 (M4) do not function as di-leucine-based motifs. As expected, incubation of anti-c-myc

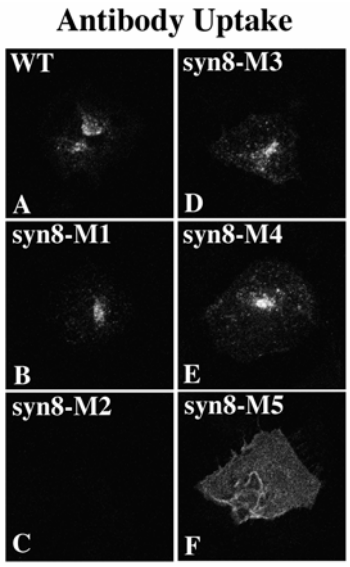

Fig. 3. Antibody uptake in di-leucine-based motif mutants. Clone 9 cells were transfected with three myc-tagged wild-type (WT) syntaxin 8, or di-leucine-based syntaxin 8 mutants (syn8-M1 to M5). Twenty-four hours after transfection, the cells were incubated with anti-c-myc antibody for 3 hours before fixing. Fixed cells were stained with the secondary antibody. Cells expressing syn8-M2 showed no staining. Cells expressing syn8-M5 were stained only at the plasma membrane.

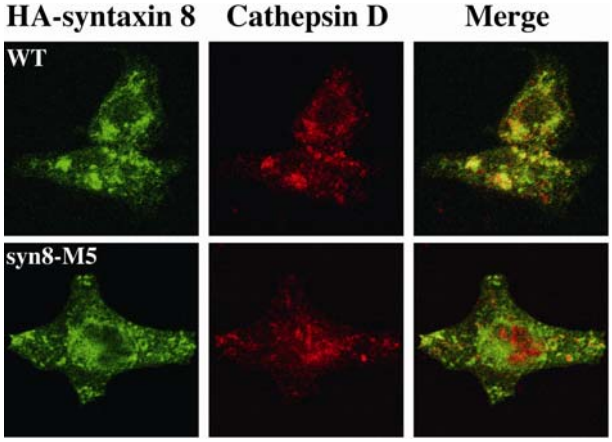

Fig. 4. Intracellular localization of the syntaxin 8 M5 mutant. Clone 9 cells were transfected with HA-tagged wild-type syntaxin 8 (WT) or syntaxin 8 M5 (syn8-M5). After incubation for 12 hours, the cells were fixed and stained with anti-HA and anti-cathepsin D antibodies. Cells expressing wild-type syntaxin 8 showed localization to cathepsin D-positive endosomal/lysosomal populations. Cells expressing syn8-M5 also co-localized to cathepsin D-positive endosomal/lysosomal populations.

antibody with cells that expressed syn8-M5 resulted in labeling only at the plasma membrane (Fig. 3F). This clearly indicates that the putative di-leucine-based motif at amino acids 184-190 (M5) functions as a di-leucine-based motif and is important for the internalization of syntaxin 8. In yeast, t-SNARE Vam3p is delivered directly from the late Golgi to vacuoles via the AP-3 dependent pathway [34]. However, the transport pathway(s) by which endosomal/ lysosomal syntaxins are delivered to their destinations are poorly understood. In this study, by determining the effects of mutation at amino acids 189-190 (M5), we found that this mutant was localized to cathepsin D-positive endosomal/lysosomal populations (Fig. 4) at a lesser degree than wild-type syntaxin 8, despite having an endocytosis defect (Fig. 3). This data suggests that syntaxin 8 may be delivered from the TGN to endosomal/lysosomal populations directly, rather than being transported to the plasma membrane and then recycled to endosomes. It is possible to think that the di-leucine-based motif at amino acids 77-83, which is involved in exocytosis from the TGN, may bind to the AP-1 or AP-3 complex and be delivered directly to endosomal/lysosomal populations.

Combined, the results indicate that syntaxin 8 has two distinct di-leucine-based motifs at amino acids 77-83 and 184-190 and that these independently function respectively for exocytosis and endocytosis. This is the first report that two di-leucine-based motifs in the same molecule act independently in distinct transport pathways.

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