研究报告

Direct Interaction With Syntaxin 1A Defines The Intracellular Localization of Munc18a*

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Abstract Syntaxin 1A (Syn1A) and Munc18a play essential roles in vesicular trafficking and exocytosis. The molecular mechanism underlying the sorting of these two proteins to their physiological sites of action remains poorly understood. Here the localization of syntaxin1A (Syn1A) and Munc18a was analyzed in baby hamster kidney (BHK-21) cells and human embryonic kidney (HEK293) cells. The rat Syn1A gene was fused to the gene encoding the enhanced green fluorescent protein (EGFP). Munc18a was labeled with the red fluorescence protein (TDimer2) at its C terminal. The proteins were expressed by transient transfection in either BHK-21 or HEK293 cells. Under fluorescence microscopy, it was shown that Syn1A was shown to be transported to the plasma membrane. While Munc18a exhibited mainly cytosolic distribution when expressed alone. However, upon coexpression with Syn1A, Munc18a is translocated to the plasma membrane. In addition, a N-terminal truncated mutant Syn1A failed to localize at the plasma membrane, suggesting that the cytoplasmic domain of Syn1A is important for its sorting and localization.

Key words syntaxin 1A, Munc18a, transportation, intracellular localization

The SNARE (soluble N-ethyl-maleimide sensitive factor attachment protein receptor) proteins play crucial roles in mediating intracellular membrane fusion in eukaryotic cells[1]. Intracellular SNAREs can be divided into two categories: the v-SNAREs located on carrier vesicles, and the t-SNAREs presented on target compartments. Many different isoforms of v- and t-SNARE proteins have been identified and localized to specific organelles[1, 2]. In order to function properly, the SNAREs must be correctly sorted and transported to their destination compartments. The mechanism of SNARE targeting in animal cells remains poorly understood. It has been suggested that SNARE sorting depends on the transmembrane domains [3]. A dileucine-based motif is thought to be required for the presence of syntaxin in endosomal compartments [4]. Accessory proteins might also modulate the trafficking of SNAREs. Rowe *et al.* [5,6] demonstrated that Munc-18a was necessary for the delivery of Syn1A from the Golgi complex to the plasma membrane. However, this hypothesis has been challenged recently by another research demonstrating that syntaxin transportation to the membrane is independent of Munc18 proteins[7].

Munc-18a is a hydrophilic protein of 68 ku devoid of membrane targeting sequences[8]. However, a pool of this protein is associated with membrane structures. Munc18a has been shown to bind the closed conformation of Syn1A from *in vitro* assays. The association of Munc-18 with the plasma membrane may thus be mediated by its interaction with membrane proteins, probably with the syntaxin family members[8,9].

In order to gain further insight into the mechanisms of the sorting and transportation of Syn1A and Munc18a, as well as the interaction of Munc18a with Syn1A, we constructed and expressed green fluorescence protein (GFP) labeled Syn1A and Munc18a in HEK293 cells and BHK-21 cells. The expression and subcellular distribution of these proteins in transfected cells were analyzed by fluorescence microscopy. Our results demonstrated that the transmembrane domain of Syn1A is insufficient to localize the protein at the plasma membrane, suggesting that its cytoplasmic domain plays important role in the sorting and trafficking of the protein. Furthermore, we showed that Munc18a is translocated to the plasma membrane via interaction with Syn1A.

1 Materials and methods

1.1 Plasmids construction

Wild type Syn1A and Munc18a cDNAs were obtained from Dr. Yuechueng Liu (University of Oklahoma Health Sciences Center, Oklahoma, USA). A 700-bp fragment containing the Syn1A open

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reading frame (ORF) was amplified with Pfu DNA polymerase (Stratagen, La Jolla, CA). The primers induced an in-frame *Bgl* Ⅱ site before the start codon and a *Kpn* Ⅰ site after the stop codon. The sequences of Syn1A primers are: forward 5′ GCGCAGATCTATGAAGGACCGAACCCAGG 3′ and reverse 5′ CCGGTACCTTATCCAAA GATGCCCCCGATG 3′. The *Bgl* Ⅱ-*Kpn* Ⅰ-digested PCR product was then ligated into *Bgl* Ⅱ-*Kpn* Ⅰ-digested pEGFP-C1 vector (Clontech Laboratories, Palo Alto, CA).

For construction of EGFP-labeled N-terminal truncated Syn1A comprising only the trans-membrane domain (Syn1A_TMD), the PCR fragment containing the trans-membrane domain was digested and ligated into *Bgl* Ⅱ-*Kpn* Ⅰ-digested pEGFP-C1 vector. The primer sequences for Syn1A_TMD are: forward 5′ GCGCAGATCTACCAAGAAGGCCGTCAAGTACC AG 3′ and reverse 5′ CCGGTACCTTATCCAAAG ATGCCCCCGATG 3′.

The plasmid pRSETB-TDimer2 was kindly provided by Dr.Tsien (University of California, San Diego). For construction of Munc18a-Tdimer2 fusion protein, the Tdimer2 fragment was digested with *Bam*H Ⅰ and *Eco*R Ⅰ, and ligated into *Bam*H Ⅰ-*Eco*R Ⅰ-digested pcDNA3.1Zeo(+) vector (Invitrogen Life Science) to produce the subclone pcDNA3. 1-TDimer2. The primer sequences for amplifying Munc18a fragment are: forward 5′ GGCGGTACC ACCACCATGGCCCCCATTGG 3′ and reverse 5′ GGCGGAGATCTGCACTACTTATTTCTTCGTCTG TTTTATTCAGC 3′. The *Kpn* Ⅰ-*Bgl* Ⅱ-digested PCR fragment was ligated into *Kpn* Ⅰ- and *Bam*H Ⅰ-digested pcDNA3.1-TDimer2.

All DNA cloning were performed using *E.coli* DH5α competent cells. Construct integrity was verified using DNA sequencing analysis provided by United Gene Holdings, LTD (Shanghai, China). Restriction enzymes and other standard molecular biology reagents were from New England Biolabs.

1.2 Cell culture

Baby hamster kidney cells (BHK-21) were grown in Glasgow's modified Eagle's medium (G-MEM) supplemented with 5% fetal calf serum (FCS; Life Technologies), 2 mmol/L L-glutamine, 10% tryptose phosphate broth, 100 U/ml penicillin, and 100 g/L streptomycin. HEK293 (Human Embryonic Kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 g/L streptomycin. All reagents for cell culture were obtained from Life Technologies (Rockville, MD, USA).

BHK-21 cells and HEK293 cells were kindly provided by Dr.Rettig (University of Homburg, Germany) and Dr.David Xu (Karolinska Institute, Stockholm, Sweden), respectively.

1.3 Transfection

BHK-21 or HEK293 Cells were transfected with Effectene Transfection Reagent kit (Qiagen) as manufacture's recommendations. 0.4 μg of the purified plasmid DNA was used for cells cultured on 96-well plates. The day prior to the experiment, cells were transferred onto a poly-L-lysine-coated round coverslip at a density of 50 000 cells per chamber.

1.4 Fluorescence imaging

Cells were growing on high refractive-index glass coverslips (n=1.78) and viewed with a fluorescence microscope system (IX70; Olympus) as described previously[10]. We took advantage of the high numeric aperture objective (APO ×100 OHR, NA = 1.65, Olympus) to take high resolution fluorescence images of transfected cells. Excitation light from a fiber optical coupled monochromator (polychrome Ⅳ; TILL Photonics GmBH, Germany) was passed through a shutter that opened only during camera exposure. The wavelength selection and switch were controlled by the image acquiring software (TILL vision 4.0; Till Photonics GmBH). Images were acquired with a cooled CCD (PCO SensiCam; Germany) with pixel size of 0.067 μm at the specimen plane. Simultaneous GFP and TDimer2 imaging was conducted by placing a Dual-View Micro-Imager (Optical-Insights) between the microscope and camera. Appropriate dichroic mirror (505 DCLP from Chroma) and emission filters were used for imaging. Images were viewed, processed and analyzed in TILL Vision (T.I.L.L.Photonics, Germany) and Adobe Photoshop (Adobe Systems).

1.5 Confocal imaging

Cells were viewed under Olympus confocal laser scanning biological microscope FV500 with Zeiss α-Plan Fluar 100× (NA=1.45) oil objective 24 hours after transfection. GFP fluorescence was excited by 488 nm Argon laser and TDimer2 fluorescence was excited by 543 nm HeNe laser (Melles Griot). Images were acquired and analyzed using FLUOVIEW (Olympus) and Photoshop 6.0.

2 Results

2.1 Construction of EGFP-Syn1A, EGFP-Syn1A_TMD and Munc18a-TDimer2

The cDNAs encoding rat Syn1A and its transmembrane domain (251~288 a.a.) were tagged at their amino termini with DNA encoding codon-optimized enhanced GFP (Figure 1), and the chimeric genes were subcloned into a mammalian

expression vector pEGFP-C1. Munc18a was fused with TDimer2 in pcDNA3.0Zeo vector. The constructs were introduced into BHK-21 cells and HEK293 cell line by transient transfection.

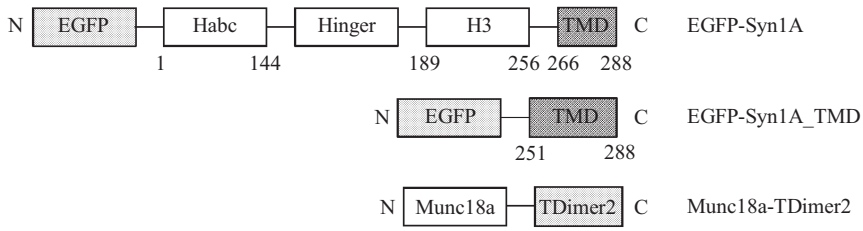

Fig.1 Illustration of the EGFP-Syn1A, EGFP-Syn1A_TMD and Munc18a-TDimer2 chimeric proteins

The Syn1A protein and the trans-membrane domain were tagged with EGFP at their amino teimini. The Munc18a protein was fused with TDimer2 at its C-terminus. The length in amino acids (a.a.) of each region is indicated. The amino (N) and carboxyl (C) termini of the fusion protein are indicated.

2.2 Localization of Syn1A at the plasma membrane

To analyze the localization of the constructed chimeric Syn1A in mammalian cells, we first transfected EGFP-Syn1A in HEK293 cells. The expression and subcellular distribution of these proteins in transfected cells were analyzed by fluorescence microscopy. Syn1A displayed a strong plasma membrane localization as well as in the perinuclear region (Figure 2a) in HEK293-transfected cells. In contrast, Munc-18a was mainly diffused in the cytosol when expressed alone (Figure 2b). Since

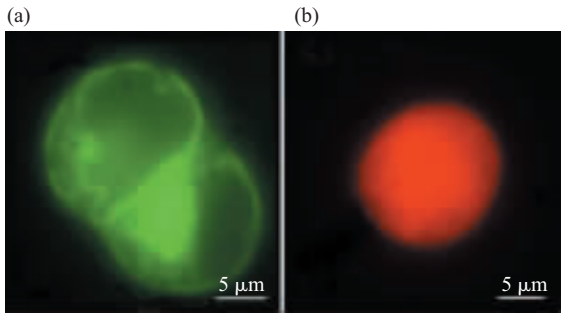

Fig.2 Distributions of exogenous expressed EGFP-Syn1A and Munc18a-Tdimer2 in HEK293 cells

EGFP-Syn1A (a) or Munc18a-Tdimer2 (b) transfected HEK293 cells were visualized by fluorescence microscopy 24 h after transfection. (a) shows the localization of EGFP-syn1A in the perinuclear region and the plasma membrane. (b) shows the diffused distribution of Mnc18a-TDimer2 in the cytoplasm.

different localization of Syn1A has been suggested for different cell types, we have utilized another cell line, BHK-21, to verify the distribution of the two proteins. When expressed alone in BHK-21 cells, Syn1A appeared mainly in perinuclear structures after 6 h of transfection (data not shown). However, at later stage of the infection (after 24 h), Syn1A developed additional membrane localization in addition to the perinuclear structures. The localization of Syn1A is similar to that of Sso2p (a yeast homologue of Syn1A) in BHK cells[11]. Munc18a displayed a homogeneous distribution in the cytosol when expressed alone in BHK-21 cells (Figure 3b).

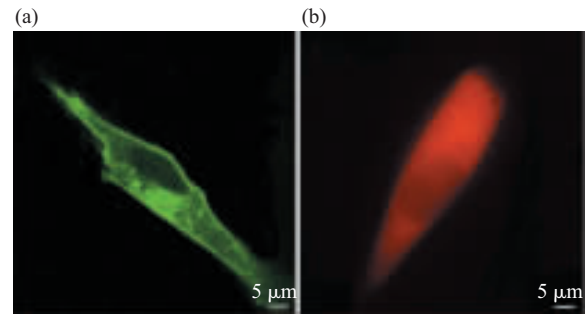

Fig.3 Distributions of exogenous expressed EGFP-Syn1A and Munc18a-Tdimer2 in BHK-21 cells

EGFP-Syn1A (a) or Munc18a-Tdimer2 (b) transfected BHK-21 cells were visualized under confocal laser scanning fluorescence microscope 24 h after transfection. (a) shows the localization of EGFP-syn1A in the perinuclear region and the plasma membrane. (b) shows the diffused distribution of Munc18a-TDimer2 in the cytoplasm.

2.3 The interaction of Syn1A and Munc18 drives Munc18a to the plasma membrane

The expression of neuronal type Syn1A and Munc18a has not been reported in nonneuronal HEK293 and BHK-21 cells. We then asked whether to coexpression of Syn1A and Munc18a could alter their localization inside the cell. As shown in Figure 4, distinct membrane localization of Syn1A was obvious after the co-transfection of Munc18a and Syn1A in HEK293 cells. However, Munc18a was no longer cytosolic localized when coexpressed with Syn1A. It rather translocated from the cytosol to the plasma membrane (Figure 4b). Merged fluorescence images of

Syn1A and Munc18a revealed that the two proteins were co-localized (Figure 4c). Similar distribution pattern was also observed in BHK-21 cells with confocal laser scanning microscope (Figure 5). Therefore, coexpression of Munc18a with Syn1A favored its localization at the plasma membrane, possibly through the direct interaction with membrane-associated Syn1A.

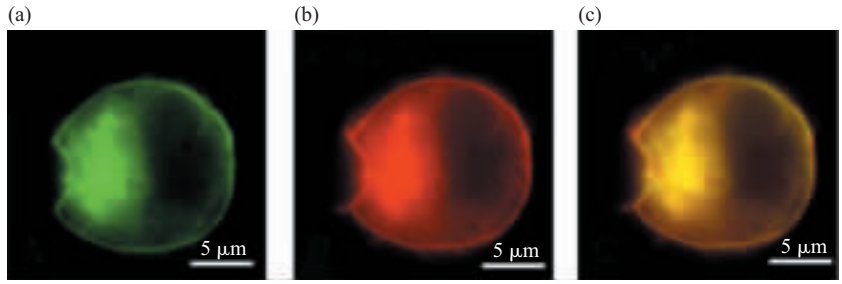

Fig.4 Translocation of Munc18a to the plasma membrane in the presence of Syn1A in HEK293 cells
HEK293 cells were co-transfected with EGFP-Syn1A (a) and Munc18-TDimer2 (b) and were visualized by fluorescence microscopy 48h after transfection. Images from (a) and (b) were merged in (c) to verify the co-localization of the two proteins.

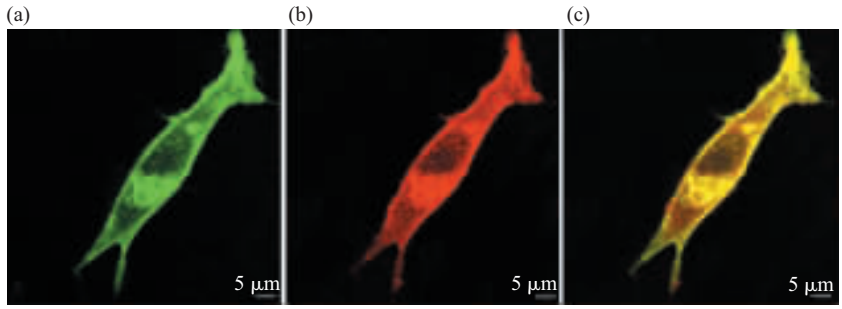

Fig.5 Translocation of Munc18a to the plasma membrane in the presence of Syn1A in BHK-21 cells
BHK-21 cells were co-transfected with EGFP-Syn1A (a) and Munc18-TDimer2 (b) and were visualized by confocal laser scanning microscopy 24 h after transfection. Images from (a) and (b) were merged in (c) to verify the co-localization of the two proteins.

2.4 The trans-membrane domain is insufficient for the trafficking of Syn1A

The C-terminal hydrophobic sequence of the tail-anchored proteins has been suggested to be responsible for the membrane anchoring [12]. To establish the role of the hydrophobic C-terminal segment of Syn1A, we generated a truncated variant of the protein (Syn1A_TMD) tagged with EGFP at the N-terminus. In contrast to the results obtained with wild type Syn1A, when expressed in HEK293 cells, EGFP-Syn1A_TMD displayed only cytoplasmic distribution with no distinct appearance on the plasma membrane. The result indicates that the EGFP-fused transmembrane domain of Syn1A is insufficient to localize the protein to the plasma membrane in HEK293 cells and that the cytoplasmic domain may play an important role in the trafficking of Syn1A. Coexpression with Syn1A_TMD fails to relocate Munc18a to the plasma membrane (Figure 6), which may further support that the trafficking of Syn1A drives Munc18a to the plasma membrane through the interaction of Syn1A and Munc18a.

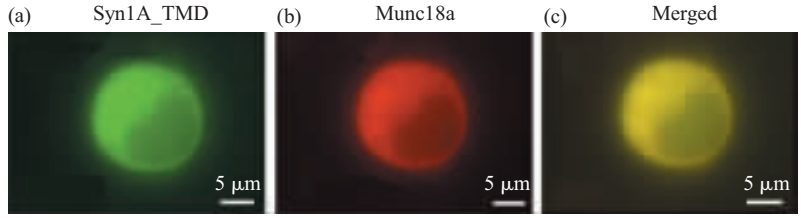

Fig. 6 Syn1A_TMD fails to locate at the plasma membrane in HEK293 cells
EGFP-Syn1A_TMD (a) and Munc18a-TDimer2 (b) cotransfected HEK293 cells were visualized by fluorescence microscopy.

3 Discussion

A number of tail-anchored proteins have been shown to be essential components of the machinery regulating the membrane traffic both in yeast and in higher eukaryotic cells[13]. The sorting and trafficking mechanism of tail-anchored proteins has largely remained obscure. In the present study, we have investigated the intracellular distribution and trafficking of Syn1A, a tail-anchored t-SNARE protein that serves as one of the core proteins in membrane fusion. Syn1A was transiently expressed in BHK-21 and HEK293 cell lines. The localization of the protein was studied *in vivo* by fluorescence microcopy. The data demonstrated that Syn1A was localized at the plasma membrane, whereas the N-terminal truncated Syn1A (Syn1A_TMD) displayed a diffused distribution in the cytosol. The different localizations of full length Syn1A and Syn1A_TMD in HEK293 cells suggests that the cytoplasmic portion of Syn1A may play an important role in the trafficking of the protein. EGFP-fused Syn_TMD did not located at the plasma membrane may due to the lack of the cytoplasmic domain of Syn1A. However, there may be another possibility that EGFP may affect the translocation of Syn_TMD. Further research on Syn1A trafficking is needed.

Structural analyses have revealed that the cytoplasmic portion of Syn1A contains two domains, Habc and H3 (or SNARE motif), both associated with the binding of Munc18[8]. Habc and H3 domains form an antiparallel four-helix bundle, which is essential for the recognition and interaction with Munc18a [14]. Munc18a is a hydrophilic protein normally distributed in the cytosol (Figure 2 and Figure 3). However, our results demonstrate that when coexpressed with Syn1A, Munc18a was also found at the plasma membrane (Figure 4 and Figure 5). When the cytoplasmic portion including the Habc and H3 domain was deleted, Munc18a failed to translocated to the plasma membrane (Figure 6). Thus, we propose Munc18a is translocated to the plasma membrane via direct interacting with the cytoplasmic domain of Syn1A.

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Syntaxin 1A 与 Munc18a 在细胞内的相互作用和定位研究 *

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摘要 Syntaxin 1A (Syn1A) 和 Munc18a 蛋白在囊泡转运和分泌中起着至关重要的作用，然而它们在细胞中分选和转运的分子机制目前尚不清楚. 我们用绿色荧光蛋白 (EGFP) 和红色荧光蛋白 (TDimer2) 分别标记 Syn1A 和 Munc18a，并用荧光显微技术观察它们在 BHK-21 和 HEK293 细胞中的转运和定位. 实验结果表明 Syn1A 主要定位在细胞质膜上，而 Munc18a 主要分布在胞浆中，但是与 Syn1A 共表达时能定位到细胞质膜上. 删除胞浆部分的 Syn1A 蛋白不能上膜，提示其胞浆结构域在分选和定位过程中起着重要的作用.

关键词 Syntaxin1A，Munc18a，转运，细胞内定位

学科分类号 Q61

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