Article

The Distribution of Synaptotagmin II in RBL-2H3 and Its Regulation on Exocytosis of Lysosomes in RBL-2H3

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Synaptotagmin (Syt) constitutes a family of membrane-trafficking proteins, so far nearly 20 Syts have been discovered. Extensive work showed that synatotagmins were a potential Ca^{2+} sensor for regulated exocytosis. This study was to investigate the expression and location of synaptotagmin II (Syt2) in RBL-2H3 (RBL) and its role in regulating exocytosis of RBL. The expression of Syt2 in RBL was confirmed by Western blot. The recombinant expression vector pEGFP-N1-Syt2 was constructed and transfected into RBL by electroporation, the stable transfectant RBL-Syt2-S expressing fusion protein Syt2-EGFP were obtained and Syt2 was highly concentrated at plasma membrane with little detected in cytoplasm. To analyze the role of Syt2 during exocytosis of RBL, the release of cathepsin D was assayed by immunoblotting. Compared with control, the release of cathepsin D by RBL-Syt2-S was markedly decreased. The results indicated that Syt2 played a negative regulation in exocytosis of lysosomes in RBL. *Cellular & Molecular Immunology*. 2005;2(3):205-209.

Key Words: calcium binding protein, mast cell, gene expression, lysosome, exocytosis

Introduction

Mast cells are specialized immune cells able to export a variety of potent protein and non-protein inflammatory mediators stored in cytoplasmic granules, which contribute to host defense and allergic responses. Upon activation, mast cells often release their populations of secretory granules, typically within a minute. Granule discharge takes place by compound exocytosis, a complex process that consists of trafficking, anchoring, priming and membrane fusion (1). While a great deal is known about the cell surface events and signaling events that transmit the stimulus to the secretory apparatus, much less is known about the distal mechanisms that mediate the final secretory processes.

In the research of mechanism about exocytosis of neuronal and neuroendocrine cells, scientists found that synaptotagmin (Syt) maybe served as calcium sensor (2). Syt is still one of hot spot in the field of exocytosis research due to its detailed mechanism unknown. Syts express not only in neuronal and neuroendocrine cells, but also in other cells including immune cells, so Syt is involved in regulation of exocytosis in neuronal cells, neuroendocrine cells, immune cells and other cells. In order to investigate the role of Syt in exocytosis of mast cells, we utilized rat basophilic leukemia cells (RBL-2H3), a mucosal mast cell line as an *in vitro* model for mast cell research. First we confirmed synaptotagmin II (Syt2) was expressed in RBL, and then we explored the location of Syt2 in RBL and its role in exocytosis of RBL.

Materials and Methods

Cell culture

RBL-2H3, a rat basophilic leukemia cell line, was maintained by passaging in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 incubator.

Plasmid and reagents

The expression vector pEGFP-N1 was purchased from Invitrogen. *E. coli* strain DH5α was stored in our laboratory. Pyrobest DNA polymerase, restriction enzymes and T4 ligase

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Abbreviations: Syt, synaptotagmin; RBL, rat basophilic leukemia cells; PMA, phorbol myristate acetate; PKC, protein kinase C; VAMP, vesicle associated membrane proteins; NSF, N-ethylmaleimide-sensitive factor; SNARE, soluble NSF-attachment protein receptor; α-SNAP, NSF attachment proteins.

were purchased from Takara. The kit for Western blot was bought from Beijing Zhongshan Company. Goat anti-rat Syt2 and goat anti-rat cathepsin D antiserum were from Santa Cruz. Fetal bovine serum was obtained from Hyclone. Ca^{2+} ionophore A23187, phorbol myristate acetate (PMA), anti-dinitrophenyl (DNP)-specific immunoglobulin E (IgE), and DNP-BSA were purchased from Sigma. Rat full-length Syt2 cDNA was kindly provided by Dr. Sudhof (University of Texas Southwestern Medical Center, USA).

Primer design

On the basis of the reported total encoding sequence of rat Syt2, the upstream primer was added an EcoR I site and the downstream primer was added a Kpn I site. The more important things for designing the downstream primer were as follows: the stop codon of Syt2 cDNA should be deleted; Syt2 and downstream EGFP gene should be kept in a same reading frame after Syt2 was inserted into plasmid pEGFP-N1. The well-designed two primers were: P1: 5'-CCC TCT GAA TTC ATG AGA AAC ATC TTC AAG AG-3'; P2: 5'-GCT ACA GGT ACC GAC TTG TTC TTG CCC AGA AG-3'. The underlined sequences indicated added EcoR I and Kpn I sites, respectively. The primers were synthesized by Shanghai Boya Company.

Detection of Syt2 expression in RBL by Western blot

RBL cells were harvested and washed twice with cold PBS, the pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml leupetin, 5 μg/ml aprotinin, 5 μg/ml chynostatin) and put on ice for 30 min and centrifugated at 15,000 g for 15 min at 4°C. The total protein in supernatant was quantitated by BSA method. Protein (30 μl) was loaded for the electrophoresis each lane, and then transferred onto a nitrocellulose filter. The membrane was blocked with 5% dry milk in TBS, incubated with goat anti-rat Syt2 antiserum, then washed three times with TBS and exposed to rabbit anti-goat IgG labled with alkaline phosphatase. After washing three times with TBS, reaction was visualized using NBT/BCIP kit. The gray scale values of the protein bands were analyzed with GDS Image Analysis System (UAP Corporation, UK).

PCR amplification

PCR was performed in a total volume of 100 μl containing 10 μl 10× PCR buffer, 2 μl dNTP (10 mM), 2 μl each primer (1 μM), 2 μl cDNA, 0.5 μl perobest DNA polymerase (5 U/μl), adding water to the final volume. PCR amplification conditions were as follows: denaturation at 94°C for 2 min for one cycle, 32 cycles of denaturation at 94°C for 45 s each, annealing at 59°C for 45 s, extension at 72°C for 2 min, and a final extension for one cycle at 72°C for 10 min. The PCR products were running in 1.2% agarose gel staining with ethidium bromide.

Construction of recombinant expression plasmid pEGFP-N1-Syt2

PCR product and pEGFP-N1 were digested with restriction enzymes EcoR I and Kpn I, then recovered with gel recovery kit and ligated with T4 ligase. DH5α was transformed with the ligased product and spread on LB plates containing 100 mg/L kanamycin. The transformants were screened and the positive clone was sent to Shanghai Boya Company for sequencing.

Cell transfection

The procedures were described as previously reported and with some modifications (3). RBL cells were harvested and counted, 5×10^6 cells were taken out and washed once with cold PBS (pH 7.4), the pellet was resuspended in 800 μl cold Optimem and transferred into an eletroporation cuvette with 4 mm gap width. The plasmid DNA (20 μg) was added in the cuvette and mixed gently. The cuvette was put on ice for 15 min. The eletroporation was performed in Gene Pulser II (BioRad) with 310 V and 950 μF capacitance. After the pulse, the cuvette was put on ice for 10 min, and then the cell suspension was pipetted into culture flask with culture medium and incubated in CO_2 incubator. After 24 h, the medium was changed and G418 (final concentration 0.8 mg/ml) was added. The clones were formed on day 10 and the positive clone was selected by limiting dilution. Stable transfection cells were harvested for Western blot analysis which was described as above.

Detection of cathepsin D

Calcium ionophore induced secretion: RBL cells were seeded in 24-well plates at 2×10^5 cells per well and incubated overnight. The cells were then washed three times in Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA) and stimulated in the same buffer with calcium ionophore A23187 (final concentration 10 μM) and PMA (final concentration 100 nM). Secretion was allowed to proceed for 30 min at 37°C. The supernatants were taken for measurements.

Antigen induced secretion: RBL cells were seeded in 24-well plates and passively sensitized by overnight incubation with DNP specific monoclonal IgE (final concentration of 75 ng/ml), washed three times in Tyrode's buffer, and then stimulated with the antigen, DNP-BSA (final concentration of 50 ng/ml). The supernatants were taken for measurements.

Detection of cathepsin D: The supernatants were concentrated in Vivaspin concentrators with a 10 kD cut-off. The concentrates were loaded to SDS-PAGE and immunoblotting with anti-cathepsin D antibodies.

Results

Western blot analysis of Syt2 expression in RBL

The protein extracted from RBL cells was separated on 6% SDS-polyacrylamide gel and electroblotted to nitrocellulose membrane, which was exposed to goat anti-rat Syt2. As shown in Figure 1, the Western blot showed a positive band with molecular weight 80 kD in Lane 2, which confirmed that

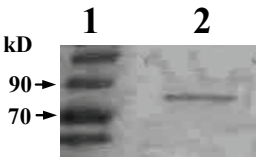

Figure 1. Western blot analysis of expressed Syt2 in RBL. Lane 1, marker; Lane 2, total cell lysate of RBL.

RBL expressed Syt2.

Identification of PCR product

Clear PCR amplified band of Syt2 cDNA was visualized in electrophoresis on 1.2% agarose gel (Figure 2). The size of amplified product was round 1.3 kb, which was in correspondence with the expected Syt2 cDNA in size.

Construction of recombinant expression plasmid pEGFP-N1-Syt2

The amplified PCR products were digested with restriction enzymes EcoR I and Kpn I, the Syt2 gene fragment was recovered and inserted into expression vector pEGFP-N1. The recombinant plasmid was constructed and digested with restriction enzymes EcoR I and Kpn I. Figure 3 presents the results of electrophoresis for vector pEGFP-N1 and recombinant plasmids digested with the restriction enzymes EcoR I and Kpn I. For the latter, there are two bands appeared in Lane 4 for the vector and the amplified products (round 1.3 kb), respectively, indicating a successful ligation of Syt2 cDNA with the vector pEGFP-N1. The plasmid was sequenced, which showed that the inserted Syt2 sequence was exactly the same with reported Syt2 in GenBank (accession number: NM012665). More importantly, the Syt2 cDNA was inserted into expression vector pEGFP-N1 in the same reading frame with downstream EGFP gene. This plasmid was named as pEGFP-N1-Syt2-S.

Obtain of stable transfection cells and distribution of Syt2 in RBL

RBL was electroporated with recombinant expression plasmid pEGFP-N1-Syt2-S and selected by G418 and limiting dilution. The stable transfection cells expressing fusion protein Syt2-EGFP were obtained and named as RBL-Syt2-S. RBL transfected with empty pEGFP-N1 was named as RBL-pEGFP. RBL-Syt2-S showed bright green fluorescence of EGFP under fluorescence microscope (Figure 4), which indicated that RBL-Syt2-S expressed fusion protein Syt2-EGFP. The distribution of Syt2 in RBL-Syt2-S can be seen from Figure 4 by observing the location of green fluorescence, which shows that Syt2 was highly concentrated at plasma membrane with little detected in cytoplasm.

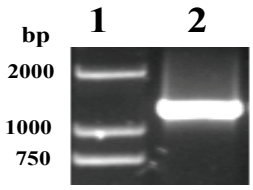

Figure 2. Analysis of PCR product. Lane 1, DL-2000 DNA size standard; Lane 2, PCR product.

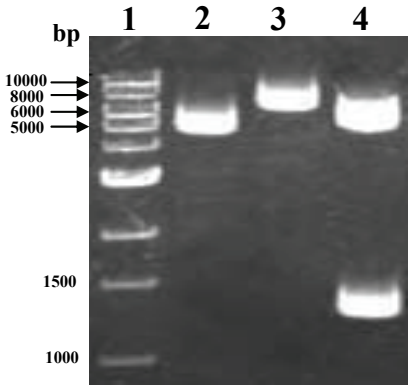

Figure 3. Restriction analysis of pEGFP-N1-Syt2. Lane 1, marker; Lane 2, pEGFP-N1 (EcoR I + Kpn I); Lane 3, pEGFP-N1-Syt2; Lane 4, pEGFP-N1-Syt2 (EcoR I + Kpn I).

Western blot analysis of Syt2 expression in stable transfection cells

Western blot was performed to analyze Syt2 expression in stable transfection cells RBL-Syt2-S and RBL-pEGFP (control). As shown in Figure 5, only one band was detected around 80 kD position for Syt2 in Lane 3, but two bands was detected in Lane 2, and one was round 80 kD for Syt2, the other was round 126 kD for Syt2-EGFP, which indicated that RBL-Syt2-S expressed not only endogenous Syt2 but also transfected fusion protein Syt2-EGFP.

Detection of cathepsin D

As shown in Figure 6, RBL-Syt2-S and control cells could not release cathepsin D without stimulator (Lane 2 and Lane 5). RBL cells could be activated to secret cathepsin D by stimulator antigen and calcium, but the latter was more effective. Compared to control (Lane 3 and Lane 4), the

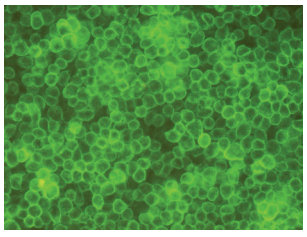

Figure 4. Location of Syt2 in RBL-Syt2-S. Syt2 was highly concentrated at plasma membrane with little detected in cytoplasm (200×).

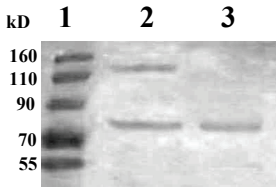

Figure 5. Western blot analysis of expressed Syt2-EGFP in RBL-Syt2-S. Lane 1, marker; Lane 2, total cell lysate of RBL-Syt2-S; Lane 3, total cell lysate of RBL-pEGFP.

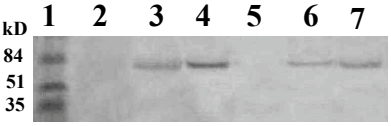

Figure 6. Detection of cathepsin D secreted by RBL-Syt2-S and control. Lane 1, Marker; Lane 2, RBL-pEGFP (no stimulation); Lane 3, RBL-pEGFP was stimulated by DNP-BSA antigen; Lane 4, RBL-pEGFP was stimulated by A23187; Lane 5, RBL-Syt2-S (no stimulation); Lane 6, RBL-Syt2-S was stimulated by DNP-BSA antigen; Lane 7, RBL-Syt2-S was stimulated by A23187.

cathepsin D secreted by RBL-Syt2-S (Lane 6 and Lane 7) decreased markedly (50% of control level), which demonstrated overexpression of Syt2 in RBL inhibited the secretion of cathepsin D in RBL.

Discussion

Syts constitute a family of membrane-trafficking proteins, so far nearly 20 Syts have been discovered (4). Structurely, Syts are characterized by an N-terminal transmembrane region (TMR), a variable linker, and two C-terminal C2-domains (C2A and C2B) similar to protein kinase C (PKC). Some Syts are localized on the plasma membrane and others on synaptic vesicles. Syts can bind to phospholipid, Ca^{2+} and syntaxin and exhibit interactions with SNAREs (*via* the SNARE protein syntaxin) (5). Extensive work showed that Syts were potential Ca^{2+} sensors for regulated exocytosis in many cell types including neuronal and neuroendocrine cells, immune cells and other cells.

Syt1 and Syt2 are the most abundant Syts which are very important among Syts in neuronal and neuroendocrine cells. In the present study, we confirmed that RBL cells endogenously expressed Syt2 at the protein level by Western blot analysis. In order to investigate the location and role of Syt2 in RBL, the recombinant expression vector pEGFP-N1-Syt2 was constructed. During transfection of the expression plasmid into RBL, lipofectamine 2000 reagents were used initially, but the transfection rate was very low, so the electroporation was adopted. After many times of trials, we found the optimal voltage was between 310-340V and the transfection rate was up to 20%-30%. After electroporation the stable transfection cells RBL-Syt2-S were obtained by G418 and limiting dilution. The distribution of Syt2 in RBL-Syt2-S could be obtained by observing the location of green fluorescence of EGFP, which showed that Syt2 was highly concentrated at plasma membrane with little detected in cytoplasm. Previous study in neuronal and neuroendocrine cells revealed that Syt2 was mainly located in cytoplasm (6). The subcellular distribution of Syt2 in RBL cells investigated by Baram showed Syt2 was located in lysosomal organelle (7). Available data demonstrate Syts are located on synaptic vesicles (or secretory granules) and plasma membrane, but one Syt is only located on secretory granules or plasma membrane. Our result showed Syt2 was located on plasma membrane and in cytoplasm (should be on lysosome considering Baram's result), so our result fully revealed the distribution of Syt2 in RBL cells. The similar report has not been found so far.

Syt has different character due to different location. Vesicular Syt has lower Ca^{2+} affinity and is more important in fast synaptic exocytosis than in endocrine exocytosis (8). Plasma membrane Syt, in contrast, have a higher Ca^{2+} affinity and may be more important for endocrine exocytosis (9). The new discovery about the distribution of Syt2 in the present study is interesting, which suggests that Syt2 is multi-functional involved in exocytois regulation, endocytosis regulation and other functions in RBL. Moreover it suggests the mechanism of Syt2 function is very complex.

It is clearly established that nearly all isoforms of synaptotagmin form homo- and hetero-oligomers *via* C2 domains (10). Although Syt is the major Ca^{2+} sensor in exocytosis, its detailed mechanisms during membrane fusion remain unknown. Now we propose a new model for Syt2 function in exocytosis of lysosomes in RBL on the basis of the distribution of Syt2 in RBL and oligomerization of Syt. The lysosomes in RBL are docked to plasma membrane, after Ca^{2+} influx into cytosol, the Syt2 located on plasma membrane and lysosomes bind to Ca^{2+} respectively, then both Syt2 combine each other *via* their C2B (11), their conformation change and twist occurs, which pull lysosomes and plasma membrane together tightly enough to catalyse membrane fusion.

Upon activation, a lot of biologically active substances stored in lysosomes of RBL can be released by degranulation. After being processed in lysosomes, the precursor form of cathepsin D is released and becomes mature cathepsin D, so cathepsin D is a marker for exocytosis of lysosomes in RBL. RBL-Syt2-S cells express not only endogenous Syt2, but also transfected fusion protein Syt2-EGFP, so they are Syt2-over-expressed cells. The present study showed that the cathepsin D secreted by RBL-Syt2-S decreased markedly compared to control, which demonstrated overexpression of Syt2 in RBL inhibited exocytosis of lysosomes in RBL. However, the detailed mechanism of Syt2 remains obscure. In the research of exocytosis mechanism in neuronal and neuroendocrine cells, scientists found some Syts potentiated exocytosis and others inhibited exocytose (12-14), which suggests that the molecular mechanism of Syt may be very complex. Thus we

postulate Syt2 may interact with other Syts and regulate exocytosis of RBL. The following work is to investigate the role of other Syts (including Syt1) in regulating exocytosis of RBL and the interactions between Syt2 and other Syts.

Acknowledgement

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References

1. Tucker WC, Chapman ER. Role of synaptotagmin in Ca^{2+}-triggered exocytosis. Biochem J. 2002;366:1-13.
2. Sudhof TC. Synaptotagmins: why so many? J Biol Chem. 2002; 277:7629-7632.
3. Ohtake H, Ichikawa N, Okada M, et al. Transmembrane phosphoprotein Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains as a negative feedback regulator of mast cell signaling through the FcepsilonRI. J Immunol. 2002;168:2087-2090.
4. Craxton M. Synaptotagmin gene content of the sequenced genomes. BMC Genomics. 2004;5:43.
5. Gerona RR, Larsen EC, Kowalchyk JA, et al. The C terminus of SNAP25 is essential for Ca^{2+}-dependent binding of synaptotagmin to SNARE complexes. J Biol Chem. 2000;275:6328-6336.
6. Marquèze B, Berton F, Seagar M. Synaptotagmins in membrane traffic: which vesicles do the tagmins tag? Biochimie. 2000;82: 409-420.
7. Baram D, Mekori YA, Sagi-Eisenberg R. Synaptotagmin regulates mast cell functions. Immunol Rev. 2001;179:25-34.
8. Fernandez-Chacon R, Konigstorffer A, Gerber SH, et al. Synaptotagmin I functions as a calcium regulator of release probability. Nature. 2001;410:41-49.
9. Sugita S, Han W, Butz S, et al. Synaptotagmin VII as a plasma membrane Ca^{2+} sensor in exocytosis. Neuron. 2001;30:459-473.
10. Desai RC, Vyas B, Earles CA, et al. The C2B domain of synaptotagmin is a Ca^{2+}-sensing module essential for exocytosis. J Cell Biol. 2000;150:1125-1136.
11. Sugita S, Shin OH, Han W, et al. Synaptotagmins form a hierarchy of exocytotic Ca^{2+} sensors with distinct Ca^{2+} affinities. EMBO J. 2002;21:270-280.
12. Tokuoka H, Goda Y. Synaptotagmin in Ca^{2+}-dependent exocytosis: dynamic action in a flash. Neuron. 2003;38:521-524.
13. Fukuda M. RNA interference-mediated silencing of synaptotagmin IX, but not synaptotagmin I, inhibits dense-core vesicle exocytosis in PC12 cells. Biochem J. 2004;380:875-879.
14. Machado HB, Liu W, Vician LJ, et al. Synaptotagmin IV overexpression inhibits depolarization-induced exocytosis in PC12 cells. J Neurosci Res. 2004;76:334-341.