Biochemical and Biophysical Research Communications **271,** 36–41 (2000)
doi:10.1006/bbrc.2000.2591, available online at http://www.idealibrary.com on IDEAL®

Rat Basophilic Leukemia Cells Express Syntaxin-3 and VAMP-7 in Granule Membranes

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Received March 21, 2000

In neuronal cells, it is generally agreed that SNARE proteins underlie the release of neurotransmitter. It is controversial, however, whether they also work functionally in the degranulation of RBL-2H3 cells because the expression of SNARE proteins has not been confirmed and the degranulation is not inhibited by tetanus toxin which cleaves one of SNARE proteins, VAMP-2. We investigated the expression and the localization of SNARE proteins including VAMP-7 which is insensitive to tetanus toxin. RT-PCR analysis showed the existence of SNARE proteins, including syntaxin-2, -3, -4, SNAP-23, VAMP-2, and VAMP-7. Experiments using GFP-conjugated proteins revealed that VAMP-7 was localized only in granule membranes, whereas syntaxin-3 was in both the plasma and granule membranes. Upon antigen stimulation, these proteins in granule membranes moved to the cell surface due to the fusion of granules with the plasma membrane. The results suggest the involvement of SNARE proteins in the degranulation of RBL-2H3 cells.

Mast cells and basophils are specialized secretory cells that play an important role in allergic inflammation (1). Their granules contain histamine, serotonin and other inflammatory mediators (2). Cross-linking of the high-affinity receptor for IgE (FcεRI) by a multivalent antigen is followed by an activation of protein tyrosine kinases, such as Lyn and Syk (3). The consequent activation of phospholipase C leads to phosphatidylinositol hydrolysis, production of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). And then, the elevation of cytosolic calcium together with the activation of protein kinase C (PKC) results in mediator release, which is the result of a membrane fusion reaction between the plasma and granule membranes and also granule membranes with each other (4–6).

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Although the cascade reactions from antigen binding to calcium influx have been well studied as described above, little is known about the proteins involved in the process downstream of calcium influx in mast cells and basophils. In neuronal cells, the membrane fusion mechanism of neurotransmitter release, which is also regulated by calcium ion, is well described by the SNARE hypothesis [soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor hypothesis] (7). It is originally launched to explain the specific targeting of proteins to various organelles (8). However, recent studies using clostridial neurotoxins (9), SNARE-deficient *Drosophila* (10) or a liposome fusion system (11) have shown that SNARE proteins are involved in the membrane fusion reaction itself. At the exocytotic release of neurotransmitter, the synaptic vesicle membrane protein VAMP-2 (vesicle-associated membrane protein-2) and the plasma membrane protein syntaxin-1 form a SNARE complex with SNAP-25 (synaptosomal-associated protein of 25 kDa) (12). Guo *et al.* showed that stimulation with Ca^{2+} or GTPγS induced relocation of SNAP-23, ubiquitously expressed isoform of SNAP-25 (13), in streptolysin-O-permeabilized rat peritoneal mast cells (RPMCs) (14), suggesting that SNARE proteins are involved in IgE-mediated degranulation. A previous experiment (15), however, showed that tetanus toxin, which cleaved VAMP-2 and inhibited exocytosis in neuronal cells (16), did not appear to inhibit the secretion of serotonin from rat basophilic leukemia 2H3 (RBL-2H3) cells stimulated by FcεRI cross-linking. Considering these results, one plausible mechanism is that a protein insensitive to tetanus toxin, like VAMP-7 (17), plays a functional role in exocytosis in mast cells. Therefore, it is crucial to examine whether or not the SNARE hypothesis is valid in mast cells.

In the present study, we have investigated the expression and the localization of SNARE proteins in RBL-2H3 cells, which are used widely for studies of exocytosis as a model of rat mucosal mast cells. We found that the cell line expressed several SNARE proteins including VAMP-7 and syntaxin-3, which can

0006-291X/00 $35.00

TABLE 1

The Primers Used in PCR for Amplification of SNARE Proteins

Target proteins[a]	Accession no.	Sizes (bp)	Primers	
			Forward[b]	Reverse[c]
Syntaxin-1B	M95735	867	GAATTCATGAAGGATCGGACTCAGGAGC	GTCGACCTACAAGCCCAGTGTCCCCC
Syntaxin-2	L20823	873	GAATTCAATGCGGGACCGGCTGC	GTCGACTCATTTGCCAACCGTCAAGCC
Syntaxin-3	L20820	870	GAATTCAATGAAGGACCGACTGGAGCA	GTCGACTTATTTCAGCCCAACGGACAA
Syntaxin-4	L20821	897	GAATTCAATGCGCGACAGGACCCA	GTCGACTTATCCAACGGTTATGGTGATGCC
SNAP-25A	AB003991	621	GAATTCAATGGCCGAGGACGCAGA	GTCGACTTAACCACTTCCCAGCATCTTTGT
SNAP-23	AF052596	633	GAATTCAATGGATGATCTATCACCA	GTCGACTTAGCTGTCAATGAGTTTC
VAMP-2	M24105	351	GAATTCAATGTCGGCTACCGCTG	GTCGACTTAAGTGCTGAAGTAAACGATGAT
VAMP-7	X92396	663	GAATTCCATGGCGATCTTTTTGCTGT	GTCGACCTATTTCTTCACACAGCTTGGC

[a] The source of all target proteins is rat except for VAMP-7 (human).
[b] *Eco*RI site is introduced at 5′-terminal for cloning.
[c] *Sal*I site is introduced at 5′-terminal for cloning.

associate with VAMP-7. Therefore, we tried to reveal the localization of syntaxin-3 and VAMP-7 in living cells using green fluorescent protein (GFP) variants, cyan and yellow fluorescent proteins (CFP and YFP). The fluorescence images demonstrated that both syntaxin-3 and VAMP-7 were expressed in granule membranes of RBL-2H3 cells.

MATERIALS AND METHODS

Cell culture. RBL-2H3 cells were cultured at 37°C in MEM supplemented with 10% FCS in a 5% CO_2 incubator. Nerve growth factor (NGF)-treated PC12 cells were cultured as previously described (18).

RT-PCR. The expression of SNARE proteins in RBL-2H3 cells was examined by RT-PCR. mRNAs were purified from RBL-2H3 cells or NGF-treated PC12 cells with a QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech) following the manufacturer's instructions. The resulting mRNAs were reverse transcribed into cDNA by Superscript IIRT (GIBCO BRL Life Technologies) and stored at −20°C until use. The primers used in PCR were shown in Table 1. The PCR products amplified with ExTaq (Takara) were analyzed on a 1% agarose gel.

Determination of rat VAMP-7 sequence. The degenerate forward primer 5′-YCGGWCAGAYTGAAGYC-3′ is designed based on the upstream region of the initiation codon of human and mouse VAMP-7 (X92396 and X96737, respectively). This and the VAMP-7 reverse primer shown in Table 1 were used in PCR. The products were cloned into pCR II vector with a TA cloning kit (Invitrogen) and sequenced with a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) and FITC labeled M13-universal primer by DNA sequencer DSQ-1000L (Shimadzu). The GenBank Data Bank of expressed-sequence tags (ESTs) was searched with the obtained sequence. Two distinct sets of clones with Accession Nos. AI535250 and AI231424 were used to determine the 22-bases sequence of 3′ end.

Plasmid construction. To express the GFP conjugated proteins in RBL-2H3 cells, the PCR products of syntaxin-3 and VAMP-7 were cloned into pECFP-C1 and pEYFP-C1 (CLONTECH), respectively, at *Eco*RI and *Sal*I sites and the sequences were verified by DNA sequencing as described above using FITC labeled primers designed for GFP variant vector instead of M13-universal primer.

Electroporation and microscopy. RBL-2H3 cells were transfected with CFP-syntaxin-3 and YFP-VAMP-7 with a Gene Pulser II electroporator (Bio-Rad). Electroporated cells suspended in culture medium were transferred to glass chambers and incubated at 37°C for 16 h. After 10 min exposure to 500 ng/ml of mouse anti-dinitrophenyl (DNP) monoclonal IgE (IgE-53-569, provided by Prof. T. Kishimoto, Osaka University), cells were washed three times in HEPES buffer [140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, 0.1 mg/ml sulfinpyrazone, 0.1% BSA, pH 7.4] and were subjected to imaging with an LSM510 confocal laser scanning microscope (Carl Zeiss). To stimulate cells, 200 ng/ml of dinitrophenylated-BSA (DNP_{10}-BSA) was added. Preparation of DNP_{10}-BSA was reported as described previously (19). Obtained images were processed and analyzed using NIH Image 1.61 and Adobe Photoshop 5.0.

RESULTS

Expression of SNARE proteins in RBL-2H3 cells. First, we investigated the expression of syntaxin isoform 1B, 2, 3, and 4 in RBL-2H3 cells by RT-PCR as shown Fig. 1a. While bands corresponding to syntaxin-2, -3, and -4 were clearly found (Fig. 1a, lanes 2–4), syntaxin-1B was not amplified with the primer pair based on syntaxin-1B (Fig. 1a, lane 1). The primer pair for syntaxin-1B successfully amplified the targeted gene with the template DNA from NGF-treated PC12 cells (data not shown) (20). Therefore, RBL-2H3 cells seemed to express little or no syntaxin-1B isoform. The sizes of PCR products were in good agreement with the expected size and the identity of PCR products was verified by restriction mapping. These results indicated that RBL-2H3 cells expressed syntaxin-2, -3, and -4.

Next, we examined the expression of SNAP-25A, SNAP-23, VAMP-2, and VAMP-7. As for SNAP-25 isoforms, SNAP-23, but not SNAP-25A, was detected (Fig. 1b, lanes 1 and 2). SNAP-25A was detected in the sample derived from PC12 cells. Bands of both VAMP-2 and VAMP-7 were also found (Fig. 1b, lanes 3

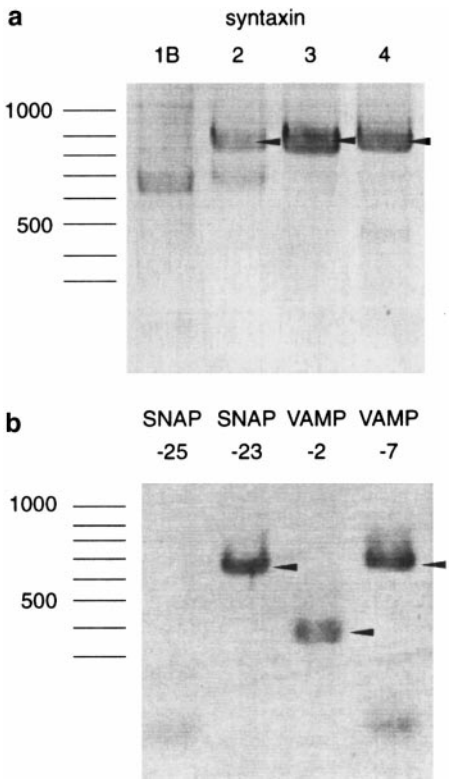

FIG. 1. Detection of SNARE proteins in RBL-2H3 cells by RT-PCR. (a) Syntaxin-1B (lane 1), -2 (lane 2), -3 (lane 3), and -4 (lane 4). (b) SNAP-25A (lane 1), SNAP-23 (lane 2), VAMP-2 (lane 3), and VAMP-7 (lane 4). The 100-bp ladder is shown from 300 to 1000 bp on the left. The expected bands are indicated with arrowheads. The expected size of syntaxin-1B and SNAP-25A is 867 bp and 621 bp, respectively, but they were not detected. In lane 1 of (a), although a weak band is found, its size is different from that of syntaxin-1B.

and 4). No PCR product was detected from the sample without reverse transcription throughout the RT-PCR experiments.

DNA sequence of rat VAMP-7. Since the sequence of VAMP-7 from rat had been unknown, we determined it as shown in Fig. 2. Comparison of the deduced amino acid sequence revealed that rat VAMP-7 had a highly similar sequence with that of human and mouse (95% identity). This conserved sequence contains heptad repeats of hydrophobic residues with Arg-151, which is necessary for the formation of a SNARE complex (21), and the altered amino acids in the tetanus toxin-sensitive sequence of VAMP-2 (17).

Intracellular localization of syntaxin-3 and VAMP-7 in living cells. We found that RBL-2H3 cells expressed syntaxin-2, -3, -4, SNAP-23, VAMP-2, and VAMP-7. With regard to syntaxin-3 and VAMP-7, further investigation was carried out. In other cells, it was reported that syntaxin-3 was involved in exocytosis (22, 23) and associated with VAMP-7 (17). Therefore, we examined the possibility that these two SNARE proteins also played a functional role in RBL-2H3 cells. To investigate the localization of syntaxin-3 and VAMP-7, these proteins were connected with CFP and YFP, respectively. Fluorescent images of CFP-syntaxin-3 and YFP-VAMP-7 were shown in Fig. 3. Before stimulation, CFP-syntaxin-3 was expressed in both the plasma membrane and granule membranes (Fig. 3d), while YFP-VAMP-7 was expressed exclusively in the granule membranes (Fig. 3g). Therefore, both syntaxin-3 and VAMP-7 resided on the organelles with a diameter of 0.5–2.0 μm. Considering the size and distribution, these organelles seemed to be secretory granules.

In addition, we tried to observe the intracellular dynamics of these two proteins when cells were stimulated by cross-linking of Fcε receptors (see Fig. 3). After addition of DNP_{10}-BSA, remarkable changes in shape of the cell and ruffling of the plasma membrane were observed in DIC images (Figs. 3a and 3b) and fluorescence images where the plasma membrane was visualized by CFP-syntaxin-3 (Figs. 3d and 3e). Granules carrying CFP-syntaxin-3 and YFP-VAMP-7 then moved to the periphery of the cell and disappeared. At the same time, the translocation of the fluorescence of granule membranes to the plasma membrane occurred. Most of the fluorescence in granule membranes was translocated in the plasma membrane within 20 min after stimulation (Figs. 3f and 3i). These dynamic changes of the localization of the fluorescence indicated the fusion reaction of granules with the plasma membrane upon activation.

DISCUSSION

As described above, we revealed the expression of several SNARE proteins in RBL-2H3 cells. VAMP-2 is known as one of SNARE proteins that play an essential role for neurotransmitter release. In neuronal cells, the treatment of tetanus toxin inhibited exocytotic release due to its proteolytic activity specific for VAMP-2 (16). On the other hand, the previous report showed that tetanus toxin did not inhibit the release of serotonin in RBL-2H3 cells (15). This suggested that VAMP-2 was not involved in the degranulation reaction although RBL-2H3 cells express it. However, this observation does not exclude the mechanism of degranulation based on the SNARE hypothesis because we found that RBL-2H3 cells expressed VAMP-7, a tetanus toxin-insensitive VAMP-2 isoform (17). The amino acid sequence of rat VAMP-7 determined in this study conserved of the coiled-coil domain with Arg-151 which is essential for the formation of a SNARE complex and exocytosis (21, 24). In addition, both the recognition sequence and cleavage site by tetanus toxin in VAMP-2 are altered in rat VAMP-7 as human VAMP-7, which is resistant to tetanus toxin (17). These features of pri-

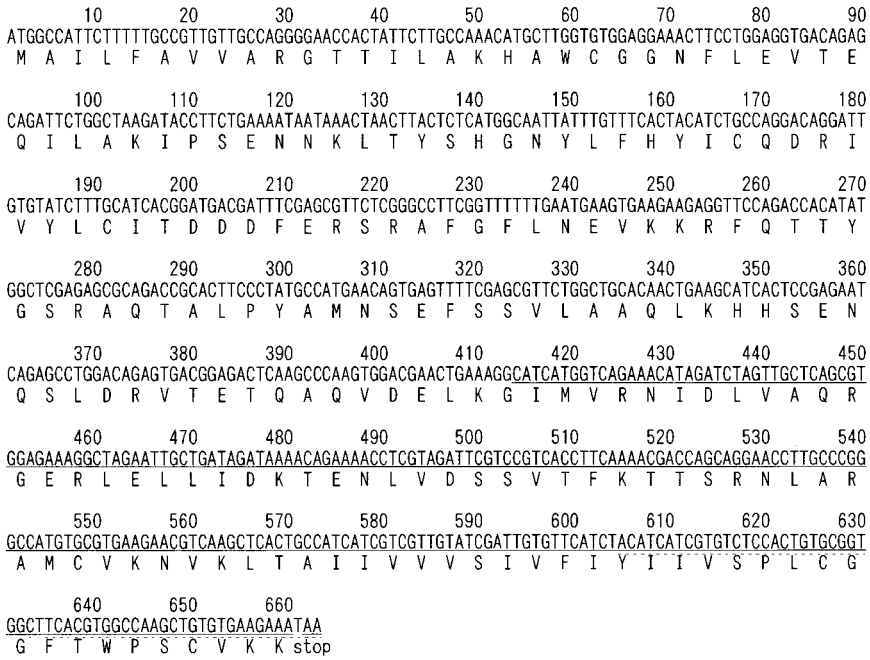

FIG. 2. DNA and deduced amino acid sequence of rat VAMP-7. DNA sequence is shown with deduced amino acid sequence in single-letter codes. DNA sequence is numbered in the 5′ to 3′ direction. Underlined regions are the sequences from EST database for determination of the 22-bases sequence of 3′ end (solid line, AI535250; broken line, AI231424) (see Materials and Methods).

mary sequence of rat VAMP-7 guarantee the structural basis for the involvement of VAMP-7 in tetanus toxin resistant degranulation. We also showed that VAMP-7 was localized in granule membranes and was translocated to the plasma membrane after antigen stimulation. In RBL-2H3 cells and RPMCs, the stimulation using phorbol myristate acetate (PMA) with calcium ionophore A23187 mimics the degranulation caused by antigen (25, 26), although PMA itself does not induce the release of mediator from granules without A23187 (27). In our preliminary experiments of RBL-2H3 cells co-transfected with CFP-syntaxin-3 and YFP-VAMP-7, the stimulation with both PMA and A23187 caused the translocation of YFP-VAMP-7 from granule membranes to the plasma membrane, whereas the stimulation with PMA only did not cause such changes. Therefore, it is strongly suggested that the SNARE hypothesis is applicable to the exocytotic release in RBL-2H3 cells.

The degranulation reaction is a fusion event between the plasma membrane and granule membranes and also among granule membranes themselves (6). The present study showed that syntaxin-3 was localized in both the plasma and granule membranes, suggesting that syntaxin-3 works as a machinery for the granule–plasma membrane fusion and the granule–granule fusion. In pancreatic acinar cells, syntaxin-3 was localized in granule membranes (28) and was served for the fusion among granules (22). In MDCK cells, syntaxin-3 and VAMP-7 were co-localized in apical route (23) and were suggested to play a role in apical traffic (23, 29). Another line of evidence obtained by immunoprecipitation using Caco-2 indicated that syntaxin-3 and VAMP-7 were able to form a complex with SNAP-23 (17). The relocation of SNAP-23 was observed in streptolysin-O-permeabilized RPMCs on stimulation with Ca^{2+} or GTPγS (14). The authors argued that this relocation was required for the degranulation reaction. Considering these results together with the data in this study, it is probable that syntaxin-3 and VAMP-7 form a fusion complex with SNAP-23, which leads to the membrane fusion reaction in the degranulation of mast cells and basophils.

It has been reported that the degranulation reaction in RBL-2H3 cells is regulated by synaptotagmin II (30) and/or Rab3d (31). Moreover, these cells also express munc18-2 (32), an isoform of munc18, which binds to syntaxin and inhibits the formation of a SNARE complex (33, 34). These proteins have been well studied in neuronal cells and play a functional role in neurotransmitter release (7, 35). This suggests that the mechanism of calcium-dependent exocytosis in these two distinct systems, degranulation of mast cells and neurotransmitter release in neuronal cells, are basically similar. Studies of SNARE proteins in neuronal cells have greatly contributed to understanding the mechanism of exocytosis and membrane fusion. Since mast cells have some advantages over nerve terminals such as their large sizes of cells and granules, the clarification of degranulation mechanism in mast cells

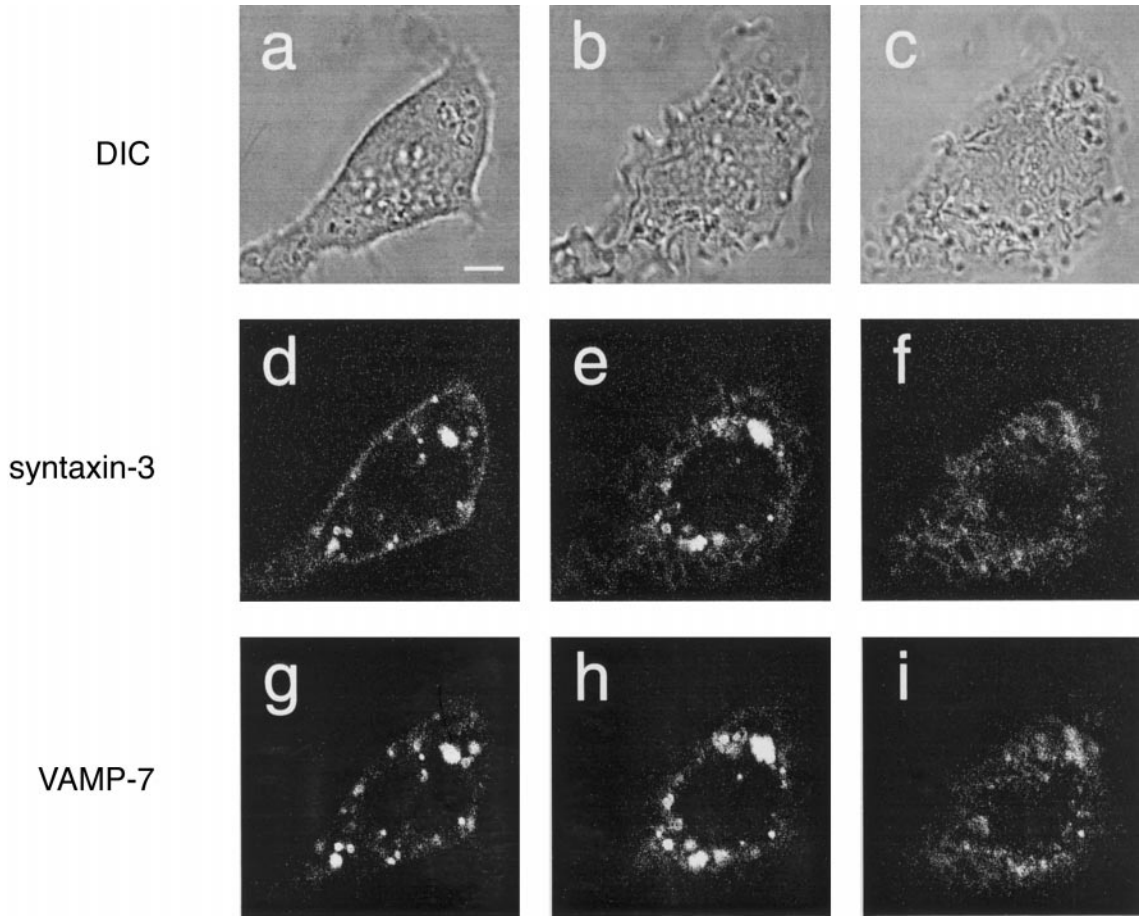

FIG. 3. Localization of CFP-syntaxin-3 and YFP-VAMP-7. (a–c) Difference interference contrast (DIC) images. (d–i) Fluorescence images of CFP-syntaxin-3 (d–f) and YFP-VAMP-7 (g–i) by confocal laser scanning microscopy (CLSM). Images obtained at 0 min (a, d, g), 3 min (b, e, h), and 21 min (c, f, i) after stimulation with 200 ng/ml of DNP_{10}-BSA are shown. Bar, 5 μm.

should provide important information regarding the basic and common mechanism of exocytosis in various secretory cells.

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