Video Article

Analysis of SNARE-mediated Membrane Fusion Using an Enzymatic Cell Fusion Assay

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Abstract

The interactions of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins on vesicles (v-SNAREs) and on target membranes (t-SNAREs) catalyze intracellular vesicle fusion¹⁻⁴. Reconstitution assays are essential for dissecting the mechanism and regulation of SNARE-mediated membrane fusion⁵. In a cell fusion assay^{6,7}, SNARE proteins are expressed ectopically at the cell surface. These "flipped" SNARE proteins drive cell-cell fusion, demonstrating that SNAREs are sufficient to fuse cellular membranes. Because the cell fusion assay is based on microscopic analysis, it is less efficient when used to analyze multiple v- and t-SNARE interactions quantitatively.

Here we describe a new assay⁸ that quantifies SNARE-mediated cell fusion events by activated expression of β -galactosidase. Two components of the Tet-Off gene expression system⁹ are used as a readout system: the tetracycline-controlled transactivator (tTA) and a reporter plasmid that encodes the LacZ gene under control of the tetracycline-response element (TRE-LacZ). We transfect tTA into COS-7 cells that express flipped v-SNARE proteins at the cell surface (v-cells) and transfect TRE-LacZ into COS-7 cells that express flipped t-SNARE proteins at the cell surface (t-cells). SNARE-dependent fusion of the v- and t-cells results in the binding of tTA to TRE, the transcriptional activation of LacZ and expression of β -galactosidase. The activity of β -galactosidase is quantified using a colorimetric method by absorbance at 420 nm.

The vesicle-associated membrane proteins (VAMPs) are v-SNAREs that reside in various post-Golgi vesicular compartments¹⁰⁻¹⁵. By expressing VAMPs 1, 3, 4, 5, 7 and 8 at the same level, we compare their membrane fusion activities using the enzymatic cell fusion assay. Based on spectrometric measurement, this assay offers a quantitative approach for analyzing SNARE-mediated membrane fusion and for high-throughput studies.

Video Link

The video component of this article can be found at <http://www.jove.com/video/4378/>

Protocol

1. Cell Culture and Transfection

1. COS-7 cells are cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 4.5 g/l glucose and 10% fetal bovine serum (FBS).
2. Plasmid transfection is done with Lipofectamine according to the manufacturer's instructions (Invitrogen).

2. Fluorescence Microscopic Analysis of Cell Surface SNARE Expression

1. The day before transfection, 3×10^4 COS-7 cells are seeded on sterile 12-mm glass coverslips (Fisher Scientific) contained in 24-well plates.
2. For v-cells in each well, 0.25 μ g of the plasmid that encodes tTA (pTet-Off, CLONTECH) is cotransfected with 0.25 μ g of the plasmids that express flipped VAMPs.
3. For t-cells in each well, 0.25 μ g of the plasmid encoding TRE-LacZ (pBI-G, CLONTECH) is cotransfected with 0.25 μ g each of the plasmids that express flipped SNAP-25 and syntaxins 1 or 4.
4. 24 hr after transfection, cells are fixed with 4% paraformaldehyde in PBS++ (PBS supplemented with 0.1 g/l CaCl_2 and 0.1 g/l MgCl_2) for 10 min, and then blocked in 10% FBS in PBS++ for 30 min.
5. The cells are incubated for 1.5 hr with the anti-Myc monoclonal antibody 9E10 (neat hybridoma culture supernatant).
6. After four washes with PBS++, the cells are incubated for 1 hr with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) at a dilution of 1:500.
7. After four washes with PBS++, the labeled cells are mounted in Prolong Gold antifade reagent (Invitrogen).

8. Confocal images are collected on an Olympus laser scanning confocal microscope. The images are processed with the Adobe Photoshop software.

3. FACS Analysis of Cell Surface SNARE Expression

1. The day before transfection, 2×10^5 COS-7 cells are seeded in each well of 6-well plates.
2. 24 hr after transfection with the flipped SNARE, pTet-Off and pBI-G plasmids, cells are fixed with 1% paraformaldehyde in PBS++ for 15 min, and then blocked in 10% FBS in PBS++ for 15 min.
3. The cells are incubated with the anti-Myc monoclonal antibody 9E10 for 60 min.
4. After three washes with PBS++, the cells are labeled with FITC-conjugated secondary antibodies (1:200 dilution) for 45 min.
5. After three washes with PBS++, the cells are scraped off the plates with a cell scraper.
6. 15,000 cells are analyzed using a FACSCalibur flow cytometer (BD Biosciences). The mean fluorescence intensity of each sample is obtained using the CellQuest Pro software.

4. Enzymatic Cell Fusion Assay

1. The day before transfection, 1.2×10^6 COS-7 cells are seeded in each 100-mm tissue culture dish, and 2×10^5 COS-7 cells are seeded in each well of 6-well plates.
2. For v-cells, 0.5 - 5 μ g each of flipped VAMP plasmid is cotransfected with 5 μ g of pTet-Off into the cells in each 100-mm culture dish. Control cells are cotransfected with the empty vector pcDNA3.1(+) and pTet-Off. To prevent the N-glycosylation of VAMPs 1, 4, 5, 7 and 8, tunicamycin (10 μ g/ml) is included in cell culture medium during transfection.
3. For t-cells in each well of the 6-well plates, 1 μ g of flipped SNAP-25 plasmid and pBI-G are cotransfected with 0.5 μ g of flipped syntaxin1 plasmid or 0.05 μ g of flipped syntaxin4 plasmid.
4. 24 hr after transfection, the v-cells are detached from culture dishes with the Enzyme-free Cell Dissociation Buffer (Invitrogen). Detached cells are counted with a hemacytometer and resuspended in HEPES-buffered DMEM supplemented with 10% FBS, 6.7 μ g/ml tunicamycin and 0.67 mM DTT.
5. 4.8×10^5 resuspended v-cells are added to each well already containing the t-cells.
6. After 6, 12 or 24 hr at 37 °C in 5% CO₂, the expression of β -galactosidase is measured using the β -Galactosidase Enzyme Assay System with Reporter Lysis Buffer according to the manufacturer's instructions (Promega). Briefly, the cells are washed twice with PBS, and then lysed in the Reporter Lysis Buffer. Cell lysates are mixed with equal volume of Assay 2 $\times$ Buffer. As a blank control, the Reporter Lysis Buffer is mixed with the Assay 2 $\times$ Buffer.
7. After 90 min, the colorimetric reaction is stopped by adding 1 M sodium carbonate.
8. Absorbance at 420 nm is measured using a HITACHI 100-40 spectrophotometer.

Representative Results

To develop a quantitative cell fusion assay, we take advantage of the strong transcriptional activation by the binding of tTA to TRE. In the absence of tTA, transcription of the *LacZ* gene in TRE-*LacZ* is silent. When tTA is present, it binds to the TRE and activates the transcription of *LacZ*. **Figure 1** shows a flowchart of the enzymatic cell fusion assay. tTA is transfected into v-cells that express v-SNARE proteins at the cell surface, and TRE-*LacZ* is transfected into t-cells that express t-SNARE proteins at the cell surface. Fusion of the v- and t-cells results in the binding of tTA to TRE, the transcriptional activation of *LacZ*, and the expression of β -galactosidase.

Figure 2A illustrates the domain structure of flipped SNARE constructs. The preprolactin signal sequence is fused to the N-termini of SNAREs. These engineered SNAREs are called 'flipped' SNAREs because the orientation of their SNARE motifs against cellular membranes is flipped. When COS-7 cells are transfected with flipped SNARE plasmids, flipped SNARE proteins are expressed at the cell surface (**Figure 2B**). Flow cytometry is used to measure the expression levels of SNARE proteins at the cell surface (**Figures 2C and D**). In order to compare their membrane fusion capacities, VAMPs need to be expressed at the same level. Therefore, we titrated and optimized the concentration of each flipped SNARE plasmid used in transfection. Flipped SNARE plasmids are transfected at the following concentrations (per 10 cm² growth area, i.e., per well in 6-well plates): VAMP1, 0.2 μ g; VAMP3, 0.5 μ g; VAMP4, 0.5 μ g; VAMP5, 0.05 μ g; VAMP7, 1.0 μ g; VAMP8, 0.1 μ g; syntaxin1, 0.5 μ g; syntaxin4, 0.05 μ g. tTA, TRE-*LacZ* and flipped SNAP-25 were cotransfected at 1 μ g per 10 cm² growth area. Under such conditions, VAMPs 1, 3, 4, 5, 7 and 8 are expressed at the same level, while syntaxins 1 and 4 are expressed at the same level at the cell surface (**Figure 2D**). As shown by confocal and phase contrast image analysis (**Figure 2E**), 80% of the COS-7 cells are transfected with flipped SNARE plasmids. Dual labeling imaging analysis (**Figure 2F**) indicates that 70% of the transfected v-cells express both tTA and flipped VAMP proteins. Because the *LacZ* gene in TRE-*LacZ* is not expressed before cell fusion, we are not able to determine the percentage of transfected t-cells that express both TRE-*LacZ* and flipped t-SNARE proteins.

The neuronal SNAREs (v-SNARE VAMP2, and t-SNAREs syntaxin1 and SNAP-25) that mediate synaptic exocytosis are used to test the feasibility of the assay. Indeed, when the v-cells expressing VAMP2 and t-cells expressing syntaxin1/SNAP-25 are combined, robust β -galactosidase expression is detected (**Figure 3**). However, when either VAMP2 or SNAP-25 is not expressed, only baseline β -galactosidase activity is detected, indicating that cell fusion and the expression of β -galactosidase rely on interactions of v- and t-SNAREs. These results indicate that the enzymatic cell fusion assay identifies fusogenic pairings between v- and t-SNAREs efficiently.

By expressing VAMP proteins at the same level at the cell surface (**Figure 2D**), we compare their membrane fusion activities. With the t-SNAREs syntaxin1/SNAP-25, VAMPs 1, 3, and 8 have comparable and the highest fusion activities, whereas VAMPs 4 and 7 have 50% and 30% lower fusion activities, respectively (**Figure 4**). In contrast, when VAMP5 is combined with the t-SNAREs, only baseline β -galactosidase activity is detected (**Figure 4**), suggesting that VAMP5 does not drive membrane fusion with the syntaxin1/SNAP-25.

Figure 1. Enzymatic cell fusion assay. tTA is cotransfected with flipped v-SNARE plasmids, and TRE-LacZ is cotransfected with flipped t-SNARE plasmids. The fusion of v- and t-cells leads to the binding of tTA to TRE and the expression of β-galactosidase, which is measured by a colorimetric method.

Figure 2. Expression of flipped SNAREs at the cell surface. (A) Domain structure of flipped SNAREs. The preprolactin signal sequence (SS) is fused to the N-termini of SNAREs, and a Myc tag is inserted between the signal sequence and the SNARE motifs (coiled-coil). (B) COS-7 cells are cotransfected with tTA and the empty vector pcDNA3.1(+) or flipped VAMP5 plasmid. 24 hr after transfection, unpermeabilized cells are stained with an anti-Myc monoclonal antibody. Representative single-slice confocal images are shown. (C and D) 24 hr after cotransfection with tTA and pcDNA3.1(+) or flipped VAMPs (v-cells), or 24 hr after cotransfection with TRE-LacZ, flipped SNAP-25 and syntaxins 1 or 4 (t-cells), unpermeabilized cells are stained with the anti-Myc antibody and analyzed by flow cytometry. (C) Representative FACS profiles of the cells transfected with pcDNA3.1(+) or flipped VAMP1 plasmid. The mean fluorescence intensity of each sample is determined using the CellQuest Pro software. (D) To express VAMPs at the same level and express syntaxins 1 and 4 at the same level at the cell surface, flipped SNARE plasmids are transfected at titrated concentrations. Shown are the mean fluorescence intensities of cell surface staining of the SNARE proteins. Error bars represent standard deviation of 4 independent experiments. (E and F) 24 hr after cotransfection with tTA and flipped VAMP5 plasmid, cells are permeabilized with 0.2% Triton X-100 in PBS++ and (E) stained with the anti-Myc antibody, or (F) dual stained with the anti-Myc monoclonal antibody and a polyclonal antibody to the TET repressor (to detect tTA). (E) Shown are representative confocal and phase contrast images. The number of transfected cells that are labeled by the anti-Myc antibody (VAMP5) and the total number of cells in the phase contrast channel are counted ($n = 60$ cells), and transfection efficiency is calculated (80%). (F) The number of transfected cells that express both VAMP5 and tTA, and the total number of transfected cells that express VAMP5, tTA or both are counted ($n = 75$ transfected cells). The percentage of the transfected cells that express both VAMP5 and tTA is then calculated (70%). Scale bars, 20 μ m. [Click here to view larger figure.](#)

Figure 3. Cell fusion depends on the interactions of v- and t-SNAREs. v-cells that express tTA and VAMP2 are incubated with t-cells that express TRE-*LacZ*, syntaxin1 and SNAP-25. After 24 h, the cells are lysed and the activity of β -galactosidase in the cell lysates is determined using a colorimetric method by absorbance at 420 nm. Only baseline β -galactosidase activity is detected when either flipped VAMP2 or SNAP-25 plasmid is omitted from transfection.

Figure 4. Comparison of fusion activities of VAMPs. Under optimized transfection conditions, VAMPs 1, 3, 4, 5, 7 and 8 are expressed at the same level at the cell surface of v-cells. 24 hr after combining the v-cells with the t-cells that express syntaxin1/SNAP-25, cell fusion is quantified using the enzymatic cell fusion assay. Error bars represent standard deviation of 4 independent experiments. ** $P < 0.01$; *** $P < 0.001$ vs. VAMP1.

Discussion

The original cell fusion assay⁶ determines SNARE-mediated cell fusion events by fluorescence microscopy. Here we describe an innovative assay that quantifies SNARE-mediated cell fusion events by activated expression of β -galactosidase and spectrometric measurement. Using this assay, we routinely analyze 15 - 20 v- and t-SNARE combinations in a single experiment. Using flow cytometry to measure SNARE expression at the cell surface, we titrate the expression levels of VAMPs and compare their membrane fusion capacities (Figures 2 and 4). Furthermore, using the enzymatic cell fusion assay, we determined the dependence of cell fusion activity on cell surface density of VAMP1 protein, and observed no cooperativity of VAMP1 protein in the cell fusion reaction⁸, suggesting that concerted action of multiple SNARE complexes is not required to fuse cellular membranes.

There are putative N-glycosylation motifs (Asn-X-Ser/Thr) in SNARE proteins. Because N-glycosylation inhibits the fusion activities of flipped SNARE proteins⁶, two approaches have been used to prevent the glycosylation of flipped SNARE proteins. First, glycosylation-site mutations are introduced into flipped SNARE constructs⁶. Second, as described in the current protocol, the N-glycosylation inhibitor tunicamycin (6.7 μ g/ml) is included in the cell fusion reaction. In addition, 0.67 mM DTT is added in the cell fusion reaction to prevent the formation of disulfide bonds among flipped SNARE proteins, which would inhibit SNARE fusion activities. Our experiments show that the addition of 6.7 μ g/ml tunicamycin and 0.67 mM DTT in cell culture medium does not interfere with the proliferation and survival of COS-7 cells. We routinely terminate the cell fusion reaction at 24 hr after combining v- and t-cells. However, significant SNARE-mediated cell fusion is detected 6 hr after combining v- and t-cells⁸. Because the enzymatic cell fusion reaction depends on the expression of β -galactosidase after cell fusion, the time course of this readout system lags the time course of membrane fusion mediated by flipped SNARE proteins.

The enzymatic cell fusion assay offers a quantitative reconstitution assay for examining the membrane fusion activities of potential v- and t-SNARE interactions in mammalian cells. By coexpressing regulatory proteins (e.g., Munc18) and SNAREs at the cell surface or by including recombinant regulatory proteins in the cell fusion reaction, this assay can be used to elucidate the regulatory mechanisms of SNARE-mediated membrane fusion. In addition, this assay should have applications in high-throughput screening for inhibitors or activators of SNARE-mediated membrane fusion.

Disclosures

No conflicts of interest declared.

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