SNAREpins: Minimal Machinery for Membrane Fusion

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1. SNARE Mediated Membrane Fusion

SNARE proteins (soluble NSF attachment receptor) are a large family of proteins (36 members in humans) that are the key elements involved in membrane fusion.
Initially SNAREs were subdivided into two groups, v-SNAREs (vesicle membrane) and t-SNAREs (target membrane), but currently they have been separated into Qa-, Qb-, Qc- and R-SNAREs depending on their structure.

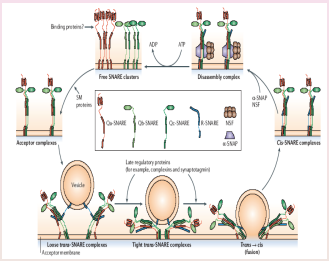

Figure 1 – Mechanism of Vesicle Docking. Jahn & Scheller, *SNAREs — engines for membrane Fusion*, Nature Reviews **7** p635.

The mechanism underlying membrane fusion is not completely understood, though there is a generally accepted model. This model states that SNARE proteins, located on the membranes that will fuse, form a four helix bundle. This bundle brings the two membranes together and presses one against the other, thus the fusion is initiated through mechanical force.

2. The Discovery of SNAREs and the Role of SNARE Cycling in Membrane Fusion

Temperature screening	1980 - 1988
VAMP Identified as important constituents of membranes….	1988 - 1992
SNAP-25,NSF are identified and U-SNARE, t –SNARE are used to rename VAMP.	1992 -1993
Discovery of // alignment of SNAREs in opposing membranes.	1997
First crystal structure of SNAREs complex determined.	1998
SNAREs directly function as catalysts and bring fusion to completion.	

Figure 2 – Timeline of SNARE research.

3. Purification and Reconstitution of SNARE Proteins into Vesicles

v-SNARE (VAMP2), and t-SNARE (syntaxin and SNAP25) were expressed in *E.coli*, and purified.

Vesicles containing the SNAREs were produced by adding phospholipids and the proteins to a octyl-*β*-D-glucopyranoside buffer. Most proteins inserted into the membrane with the cytosolic domain outside, proved by assaying with protease. v-SNARE vesicles have approximately 750 copies of VAMP2, t-SNARE vesicles have approximately 75 copies of the t-SNARE complex.

To test for fusion, vesicles were incubated together, and botulinum toxin D added to examine the amount of VAMP resistant to cleavage (see figure 5), VAMP cannot be cleaved if fused to t-SNAREs.

4. An Experiment to see if SNARE Proteins can be Exclusively Responsible for Vesicle Fusion

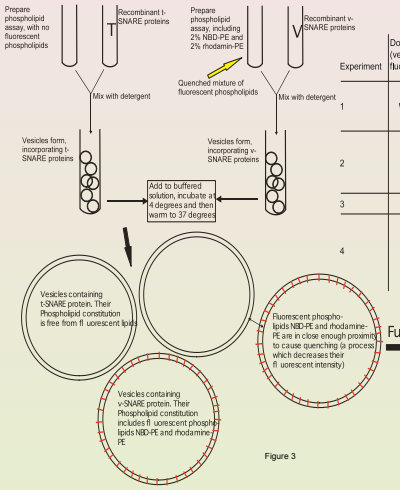

Figure 3

Experiment	Donor Vesicles (vesicles with fluorescence)	Acceptor Vesicles (no fluorescence)	Increased Fluorescence to suggest fusing?	Conclusions and Suggestions
1	With v-SNARE	With t-SNARE	YES	v-SNARE and t-SNARE form complexes to encourage vesicle fusion
2	v-SNARE	v-SNARE	NO	A control experiment, suggesting that v-SNARE vesicles can only fuse with t-SNARE vesicles (v-SNARE and t-SNARE are complimentary
3	v-SNARE	no proteins	NO	Control experiment
4	t-SNAREs	v-SNARES	YES, but slightly reduced fluorescence compared to exp 1	As suggested in previous experiments, t-SNAREs do not occupy bi-layers in such numerous amounts as v-SNARE so there is less binding potential for t-SNARE vesicles

5. Evidence for a Complete Mixing of the Lipid Bilayers

When a fusion of membranes occurs, the lipids mix with one another. Different suggestions have been made about how this mixing happens. The paper considers this question by attaching a fluorescent head to the vesicle lipids.
Dithionite is used on the vesicle to convert the outer fluorescent heads to non-fluorescent, but Dithionite does not cross lipid bilayers - resulting in a vesicle with the inner layer still fluorescent.

Experiment a:
Vesicles (labelled as above) were incubated with unlabelled target SNARE containing acceptors. Fluorescence increased, showing that lipid mixing with the acceptor population had occurred. In contrast, when vesicles were incubated with unlabelled target non-SNARE containing receptors, no increase occurred – No fusion had taken place.

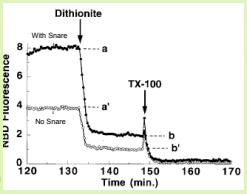

Figure 4 – SNARE mediated Fusion leads to complete mixing of bilayers.

Experiment b:
Once fusion has taken place, the question remains of whether any of the (originally inside-vesicle) fluorescent lipids remain on the inside of the new membrane. Dithionite was added to the fused membranes, to de-fluoresce the outer membrane.
Later, TX-100 was added in order to expose the inner layer to the Dithionite – resulting in the elimination of fluorescence. By way of comparison, the de-fluorescing in the inner layer $\frac{b}{b'}$ is equal to the de-fluorescing in both the inner and outer layer $\frac{a}{a'}$ – thus establishing that both layers participate in fusion to the same extent.

6. Assembly of SNARE Complexes Between Vesicles is Required for and Precedes Fusion

Four main experiments indicate that SNARE complexes form prior to any vesicle fusion events occur. Furthermore they also corroborate the hypothesis that SNARE complexes are the minimal machinery required for membrane fusion. Detailed in the table are the experiments that imply this:-

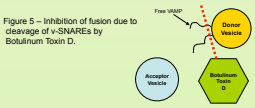

Figure 5 – Inhibition of fusion due to cleavage of v-SNAREs by Botulinum Toxin D.

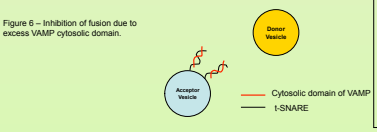

Figure 6 – Inhibition of fusion due to excess VAMP cytosolic domain.

Experiment	Procedure	Explanation
1	Incubate acceptor and donor vesicles at 4°C prior to measuring fusion (via fluorescence) at 37°C. The control for this experiment is a non-incubated sample run at 37°C.	No significant fusion occurs at 4°C however SNARE complexes can form at this temperature as observed by increase in the rate of fusion at 37°C as compared with the control. N.B. there is a negligible difference in the starting amount of fusion (near to zero fluorescence) between the preincubated sample and the control.
2	Extending the previous experiment by preincubating for several more hours.	An increased initial rate of fusion is observed when preincubation has been extended significantly. This indicates that prefusion intermediates have accumulated in the medium.
3	Add botulinum toxin D after preincubation. Control is to add botulinum before preincubation.	Botulinum toxin D cleaves uncomplexed VAMP (Fig.5) but not VAMP in a complex. As there is no significant decrease in the initial rate of fusion upon adding this toxin it is clear that free VAMP does not take part in membrane fusion.
4	Add the cytoplasmic part of VAMP after preincubation. Control is to add the above before preincubation.	The cytoplasmic domain of VAMP can act as a membrane fusion inhibitor yet when this is added there is no decrease in the initial rate of fusion whereas when this is added to solutions containing t-SNARE acceptor vesicle prior to preincubation membrane fusion is negated. This confirms that free t-SNAREs like v-SNAREs are required for assembly of prefusion intermediates but not for fusion itself

7. Conclusions

The various experiments performed in the paper support all of the hypotheses regarding membrane fusion and SNAREs. Furthermore, the experiments show that both leaflets of the bilayer participate in the fusion process to the same extent. In vitro, most SNARE proteins reconstitute in artificial vesicles in the correct orientation for membrane fusion to occur. SNAREs function as fusion catalysts in that they provide the energy for fusion and also bring fusion to completion, and SNARE complexing **must** take place before membrane fusion can occur. This paper supports the proposal that vesicles can fuse using only t-SNAREs and complimentary v-SNAREs, independent of any other proteins.

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Based on the paper by: Weber et al; SNAREpins: Minimal Machinery for Membrane Fusion, Cell, Vol. 92, 759–772, March 20, 1998