Evolution and Specificity in SNARE-mediated Vesicular Fusion
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Background:
Membranes are fundamental to the compartmentalization of biological organisms. Phospholipid bilayer segregation of biochemical machinery generates the basic topology of diffusion-connected spaces in cells. The merger of vesicles is energetically unfavorable, but once a pore connecting the internal volumes of two vesicles is created, the membrane will move towards a new energy minimum that typically expands the pore and causes the complete merger of the two vesicles [1].

A critical question that arises in eukaryotes is how membrane fusions are performed selectively in space and time. Secretory vesicles must be able to fuse with the outer membrane of the cell, yet at the same time must avoid fusing with organelles and other vesicles en route to the extracellular environment. Similarly, synaptic vesicles must avoid membrane fusion until critical signaling events in a neuron are detected. Several proteins are implicated in the system that manages vesicular fusion specificity.

In particular, the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) superfamily confers much of this in vivo specificity. Most proteins in this >100 member family are approximately 35 kDa, membrane-associated via a single C-terminal anchor, and all share a homologous conserved 60aa domain (the SNARE domain) singularly responsible for binding to other SNARE proteins. SNARE proteins can be classified into t-SNARE proteins associated with target membranes and v-SNARE proteins associated with vesicular membranes. Upon binding, the SNARE proteins form a four-helix complex. It is postulated that this complex, perhaps with the aid of helper proteins, physically bring the fusing membranes close enough to form a pore. Other proteins, including Rab and Sec1/Munc18 (SM proteins) are implicated in later steps of membrane fusion [2,3,4,5,6,7]. SNARE proteins promiscuously form complexes in vitro and in cis- on the same membrane, but are only functionally active with their cognate v-SNARE or t-SNARE partner in trans- on an opposing membrane [8]. Two angstrom crystal structures and NMR solution structures are available for selected members of the SNARE family [9,10,11].

Specific Aim 1: Develop a high-throughput assay for testing cognate SNARE fusion activity

In order to study SNARE protein interactions and analyze details of the specificity of cognate pairing, measurements of promiscuous function on non-cognate SNAREs must be performed. Previous work has focused on recombinant expression and purification of SNARE proteins for vesicular reconstitution in vitro. The need to purify each individual protein is an unbearable cost for large-scale assays and studies of SNARE proteins.

Hu et al. showed fusion of whole cells in human tissue culture via recombinant expression of SNARE proteins retargeted to the cell membrane [12]. I propose developing a high-throughput assay for SNARE fusion activity by converting this system for use in yeast. Yeast is a simple and efficient system to transform compared to human tissue culture. Given a sequence for each SNARE protein of interest, S. cerevisiae should be able to express them on their surface. By combining cultures of S. cerevisiae expressing cognate proteins, I hope to demonstrate SNARE-mediated yeast-yeast fusion.

This cell-cell fusion can be purposed for a high throughput assay by growing one of the two yeasts in the presence of X-gal (5-bromo-4-chrolo-3-indolyl-β-D-galactoside). X-gal will convert into a dark insoluble precipitate in the presence of β-galactosidase (LacZ). If the other yeast has a constitutively expressed copy of LacZ, a fusion event should increase the darkness of the solution in a predictable way. Thus, a colorimetric assay for SNARE kinetics can be developed. Cognate SNARE proteins would be expected to fuse cells at a drastically higher rate than non-cognate SNARE proteins.

Specific Aim 2: Generate a library of novel orthogonal SNARE cognate pairs
With a high-throughput assay, a number of experiments become immediately possible. First among them is assessing the selectivity of different SNARE proteins. How specific is the recognition between different
NARE proteins? This can be answered via recombinant expression of a large number of known SNARE proteins and testing via cell-cell colorimetric fusion assay.

Via mutagenesis, sensitivity of the 16 residue binding motif of SNARE proteins to mutation can be analyzed [13]. With computational analysis, the total number of possible SNARE cognate pairs with a certain specificity can be estimated and compared to the natural numbers of SNARE proteins in sequenced organisms. By mutating pairs of SNARE proteins simultaneously at a high error rate with error-prone PCR and screening for maintenance high-quality fusion kinetics, sequence-divergent SNARE cognate pairs can be developed via directed evolution.

Repeating the specificity assays with these new SNARE cognate pairs can establish whether or not they maintain specificity just for one another and lose fusion activity on parent cognates. This process will be iterated until a small number of new orthogonal SNARE cognate pairs with similar kinetics to natural SNARE proteins have been developed. The quality of these new SNARE cognate pairs can be evaluated via SNARE knockout and functional recovery in yeast.

These new SNARE cognate pairs will help both understand the way specificity is conferred and evolved. Further, they may have uses for control of membranes in new synthetic biology efforts. Membrane control in synthetic biology is thus far limited exclusively to light-activated Rab GTPase recruitment of actin, deforming a cell in response to patterned light [14]. Orthogonal SNAREs will add to the repertoire of synthetic biology tools.

References:
8) Bethani et al. The specificity of SNARE pairing in biological membranes is mediated by both proof-reading and spatial segregation. EMBO (2007)