SNARE Interactions Are Not Selective

IMPLICATIONS FOR MEMBRANE FUSION SPECIFICITY

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The SNARE hypothesis proposes that membrane trafficking specificity is mediated by preferential high affinity interactions between particular v (vesicle membrane)- and t (target membrane)-SNARE combinations. The specificity of interactions among a diverse set of SNAREs, however, is unknown. We have tested the SNARE hypothesis by analyzing potential SNARE complexes between five proteins of the vesicle-associated membrane protein (VAMP) family, three members of the synaptosome-associated protein-25 (SNAP-25) family and three members of the syntaxin family. All of the 21 combinations of SNAREs tested formed stable complexes. Sixteen were resistant to SDS denaturation, and most complexes thermally denatured between 70 and 90 °C. These results suggest that the specificity of membrane fusion is not encoded by the interactions between SNAREs.

Membrane compartments in eukaryotic cells are in constant dynamic flux. Transport vesicles emerge from donor compartments and are specifically targeted to acceptor compartments where they deliver cargo through membrane fusion (1). The ability of transport vesicles to specifically recognize appropriate acceptor membrane targets underlies the organization of the secretory pathway (2–5). The SNARE hypothesis proposes that membrane target recognition can be attributed to the specific pairing of SNARE proteins (6, 7). SNAREs, which are believed to mediate aspects of vesicle docking, priming, and membrane fusion, are common to vesicular trafficking in organisms as diverse as yeast and humans (8). SNARE proteins belonging to the VAMP (synaptobrevin), SNAP-25, and syntaxin families form heteromeric complexes that consist of extended, parallel four-helical bundles (9, 10). Interestingly, a set of absolutely conserved polar residues is buried at the center of the helical bundle (9, 11). VAMP contributes a single helix to the structure with a central, buried arginine residue. Syntaxin contributes one helix and SNAP-25 provides two helices, each contributing a buried glutamine residue. Syntaxin and VAMP are anchored to opposite membranes through single transmembrane-spanning helices. Thus, formation of a stable SNARE complex brings membranes into direct opposition and may result in membrane fusion or an activated docked state (12–14). Following membrane fusion, the SNARE complex is dissociated through the action of α-SNAP and the ATPase NSF, allowing the proteins to recycle (15, 16). The structure of the SNARE complex suggests an attractive model whereby specificity could be generated through specific helical interactions.

If SNARE complex formation is responsible for the specificity of membrane fusion in mammalian cells, several criteria must be met. First, there should be a sufficient number of SNARE proteins to account for constitutive membrane trafficking as well as the specialized and regulated secretory processes found in differentiated cell types. Second, the localization of arginine and glutamine classes of SNAREs (r-SNARE and q-SNARE, respectively) should make possible the formation of proper four-helix bundle structures across opposing membranes. Finally, trafficking pathways should be accounted for by the specificity of SNARE pairing. A compilation of SNARE proteins localized within the secretory pathway of a typical eukaryotic cell is depicted in Fig. 1. Both r- and q-SNARE proteins are specifically localized to a variety of membrane compartments throughout the secretory pathway (17–22). The number and localization of SNARE proteins indeed suggest that high affinity interactions between sets of SNAREs could account for aspects of the specificity of membrane organization. Therefore, only the final criterion remains: do SNAREs interact specifically?

We have investigated the specificity of SNARE interactions by testing the ability of the core, interacting domains from a diverse set of SNARE proteins to form stable SNARE complexes. Every combination that included a syntaxin, VAMP, and SNAP-25 family member formed a highly stable SNARE complex. These results suggest that the core interactions between SNAREs are not the source of specificity in membrane trafficking.

EXPERIMENTAL PROCEDURES

Plasmids and Plasmid Construction—Plasmids encoding the syntaxin-IA H3 domain (amino acids 191–266), SNAP-25 N-terminal domain (amino acids 1–92) and C-terminal domain (amino acids 141–206), VAMP2 cytoplasmic domain (amino acids 25–94), VAMP7 cytoplasmic domain (amino acids 1–182), and VAMP8 cytoplasmic domain (amino acids 2–75) were prepared as described previously (10, 17, 18). DNA fragments encoding the syntaxin-4 H3 domain (amino acids 200–270), syntaxin-13 H3 domain (amino acids 170–243), SNAP-23 C-terminal domain (amino acids 148–211), SNAP-29 N-terminal domain (amino acids 50–132), SNAP-29 C-terminal domain (amino acids 198–258), VAMP4 (amino acids 2–115), and rSec22b C-terminal domain (amino acids 126–195) were amplified using the polymerase chain reaction (PCR) and subcloned into the pGEX-KG vector (24). A plasmid encoding the N-terminal domain of SNAP-23 (amino acids 1–78) was

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† The abbreviations used are: SNARE, SNAP receptor; SNAP, soluble NSF attachment protein; NSF, N-ethylmaleimide-sensitive factor; SNAP-23, -25, -29, synaptosome-associated protein of 23, 25, and 29 kDa; r-SNARE, arginine class of SNARE; q-SNARE, glutamine class of SNARE; VAMP, vesicle-associated membrane protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PCR, polymerase chain reaction.
RESULTS AND DISCUSSION

If r- and q-SNARE interactions impart specificity in trafficking, specific combinations of SNAREs should form stable complexes whereas others should not. To test this hypothesis, we took advantage of the observation that syntaxin-1A, SNAP-25, and VAMP2 form a highly stable ternary complex that is resistant to SDS denaturation (12). To simplify the interpretation of our results, we focused on the interacting SNARE domains previously identified by mutagenesis and proteolytic mapping (26, 27). This complex, which can be dissociated by α-SNAP/NSF, comprises the C-terminal H3 region of syntaxin-1A (amino acids 191–266), the VAMP homologous region (amino acids 25–94), and two separate domains from SNAP-25 (the N-terminal domain, amino acids 1–82, and the C-terminal domain, amino acids 141–206). Using the separate domains of SNAP-25 eliminates higher order multimerization that might complicate binding measurements (26). Also, the potential complication of the N-terminal domain of syntaxin regulating the kinetic rate of SNARE association is eliminated (28).

Importantly, however, the N-terminal domain of syntaxin and the other protein regions not included in the analysis do not contribute to the associated SNARE complex structurally or thermodynamically (26–28). For example, thermal unfolding of the complex containing the full-length syntaxin can be deconvoluted into two separate unfolding events, one representing the N-terminal domain of syntaxin and the second representing the melting of the core SNARE complex. Furthermore, the thermal stability of the core complex is virtually identical to the complex formed using the full cytoplasmic regions, indicating that regions outside the interacting domains do not contribute to the SNARE interactions and therefore do not contribute to specificity. SDS-resistant SNARE complexes between syntaxin-1A, SNAP-25 (N- and C-terminal domains), and VAMP2 require all four domains. Any combination of three of the four domains is not sufficient to confer SDS resistance (Fig. 2A). These results attest to the importance of each of the four SNARE helical regions in forming a highly stable association.

Using this SDS resistance assay, we were able to test the ability of various r-SNAREs to substitute for VAMP2 in forming stable complexes. VAMP4, -7, and -8 and rSec22b were chosen as representative r-SNAREs that localize to various cellular compartments (Fig. 1) and that should, according to the SNARE hypothesis, interact with only specific q-SNAREs. Surprisingly, we found that all four VAMPs were able to confer SDS resistance (Fig. 2B).

These results imply that there is little selectivity in the interactions between r- and q-SNAREs. Nevertheless, it is possible that in synaptic exocytosis the q-SNARE complex, syntaxin-1A and SNAP-25, is exceptional in its ability to bind multiple VAMPs. To test this possibility, two additional q-SNARE complexes were chosen for analysis. The combination of syntaxin-4 and SNAP-23 was selected because of their ubiquitous, non-neuronal expression (19, 29), whereas the combination of syntaxin-13 and SNAP-29 was chosen because of their higher sequence divergence relative to syntaxin-1A and SNAP-25, respectively (17, 18). Like the synaptic exocytosis complex, syntaxin-13 and SNAP-29 were able to form SDS-resistant complexes with every VAMP tested (data not shown). The syntaxin-4-SNAP-23 complex, however, only formed SDS-resistant complexes with VAMP4 and VAMP7. Twelve of the 15 complexes tested were SDS-resistant, demonstrating that an overwhelming number of SNARE combinations form extremely stable structures. Thus, in contrast to the SNARE hypothesis, SNARE interactions are highly promiscuous.

To gain a more quantitative understanding of the relative stabilities of SNARE complexes, we used circular dichroism to

Fig. 1. Localization of SNARE proteins within the secretory pathway of a typical eukaryotic cell. r-SNAREs are colored red. q-SNAREs from the syntaxin family are blue. q-SNAREs from the SNAP-25 family are light blue. Other q-SNAREs are black. The SNARE proteins used in this study are underlined (see text for references). N, nucleus; ER, endoplasmic reticulum; IC, intermediate compartment; TGN, trans-Golgi network; V, vesicles; DCV, dense core vesicles; EE, early endosome; LE, late endosome; L, lysosome; CV, clathrin-coated vesicles.

generated by introducing a premature stop codon using PCR-based mutagenesis. All coding regions were sequenced to confirm the absence of PCR-induced errors.

Protein Expression and Purification—With the exception of the N-terminal domain of SNAP-25, which was expressed with a N-terminal 6-histidine tag (His₆) using the pQE-30 vector system (Qiagen), all recombinant proteins were expressed as GST fusions from the pGEX-KG vector (24). The His₆-tagged protein was expressed in Escherichia coli strain M15[pREP4] and purified using nickel-nitrilotriacetic acid tagged mixtures of the SNARE proteins were solubilized in SDS containing stable complexes. VAMP4, -7, and -8 and rSec22b were chosen as representative r-SNAREs that localize to various cellular compartments (Fig. 1) and that should, according to the SNARE hypothesis, interact with only specific q-SNAREs. Surprisingly, we found that all four VAMPs were able to confer SDS resistance (Fig. 2B).

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VAMP4, -7, or -8 all had similar $T_m$ of the unfolding transition. Under the conditions used for our measurements, the midpoint $T_m$ of the unfolding transition was measured using circular dichroism. Graphs are shown representing the fraction of unfolded complex for SYN1-SNAP-25 (A), SYN4-SNAP-23 (B), and SYN13-SNAP-29 (C), in combination with the various r-SNAREs (circles, VAMP2; squares, VAMP4; diamonds, VAMP7; triangles, VAMP8; inverted triangles, rSec22b).

Finally, we examined the effect of varying the q-SNARE on complex stability. With the r-SNARE VAMP2 constant, we tested the nine q-SNARE combinations for SDS resistance. All the combinations that included SNAP-25 or SNAP-29 formed SDS-resistant complexes (Table II); only the SNAP-23-containing complexes were not SDS-resistant. It is interesting that SNAP-23 and SNAP-25 display different SDS resistance properties, as they are highly conserved in their interacting domains (67% identical), whereas SNAP-23 and SNAP-29 are only 32% identical and SNAP-25 and SNAP-29 are only 33% identical. In terms of thermal stability of all the q-SNARE combinations only the syntaxin-13-SNAP-23 pair was particularly unfavorable (Fig. 4, A–C; Table II). Indeed, complexes containing syntaxin-13 in combination with SNAP-23, -25, or -29 were in general the least stable, with an average melting temperature of 66 °C compared with 83 and 84 °C for complexes containing syntaxin-1A and -4, respectively (Table II). It is not obvious from the sequences or the crystal structure why the syntaxin-13-SNAP-23-VAMP2 complex is less stable (9). For example, many of the salt-bridge interactions are conserved. There are, however, a few changes in the core residues,
such as an alanine (SNAP-23) to valine (SNAP-25) substitution, that could result in destabilizing side chain packing defects.

Importantly, from these data we can conclude that the stability patterns are not consistent with protein localization and known trafficking pathways. For example, rSec22b is the r-SNARE responsible for endoplasmic reticulum and Golgi trafficking, yet it forms stable complexes with combinations of syntaxins-1, -4, and -13 and SNAP-23, -25 and -29, q-SNAREs that function in post-Golgi trafficking. Furthermore, syntaxin-13 functions in recycling of plasma membrane proteins and is localized to the tubular vesicular endosomes, and syntaxin-4 is localized to the plasma membrane where it functions in exocytosis (30, 31). These q-SNAREs do not readily differentiate between the various VAMPs tested.

Why are all the SNARE complexes so stable? The activation energies for steps in vesicle fusion are reported to be large (32). If SNARE complexes function to catalyze a step in membrane fusion, the energy from complex formation could help to overcome the activation barrier encountered in bringing membranes into close proximity or perhaps catalyzing some aspect of phospholipid rearrangement (33, 34). The yeast exocytosis complex, Sso1-Sec9-Snc2p, under similar conditions to ours thermally unfolds at 72 °C (28, 35). This temperature is similar to the lower range we observed for the mammalian SNARE combinations, suggesting that most of the complexes in this study would be energetically suitable for function. The necessity for high stability in SNARE complexes perhaps is achieved at the expense of specificity.

The fact remains, however, that only certain SNARE complexes are formed in vivo. If specific SNARE associations are not thermodynamically controlled, then how are the SNARE interactions regulated? It is clear that most SNARE proteins are localized to distinct compartments in the cell, so presumably their interactions in vivo are spatially restricted. Indeed, in yeast, a mislocalized q-SNARE, Vam3p, can functionally substitute for Pep12p (36). Other sources of trafficking specificity may come from regulatory factors such as the Ypt/Rab and Sec1 families (37, 38). Rab effector proteins may function as membrane-specific docking factors, thus directing the formation of certain SNARE combinations. Also, targeting complexes, such as has been suggested for the sec6-sec8 complex, could play an important role in defining transport destinations (39). In addition, the transport of vesicles from donor to acceptor site is highly specific and likely regulated by distinct sets of motors and cytoskeletal elements. Thus, perhaps membrane fusion specificity is not due to a single mechanism but rather involves several different layers of regulation.

If SNARE interactions are not specific, why are there so many SNAREs? Distinct SNAREs are likely necessary to maintain and regulate the complex intracellular compartmental organization. Membrane trafficking is extremely dynamic and, thus, all membranes need to maintain appropriate concentrations and arrays of SNARE proteins for membrane fusion events to occur. This is accomplished through numerous antro- and retrograde trafficking pathways, which may require specific sequence information outside the core interacting regions. Such a mechanism allows the possibility of integrating multiple trafficking pathways through promiscuous SNARE pairings, as has been suggested by the finding that a single r-SNARE functions with different q-SNAREs in directing

### Table I
Thermal stabilities of SNARE complexes

| Syntaxin-IA • SNAP-25 | Syntaxin-4 • SNAP-23 | Syntaxin-13 • SNAP-29 | Average |
|-----------------------|----------------------|-----------------------|---------|
| VAMP2                 | 90°                  | 80                    | 70°     | 80      |
| VAMP4                 | 89°                  | 87°                   | 76°     | 84      |
| VAMP7                 | –92°                 | 88°                   | –85°    | 88      |
| VAMP8                 | 87°                  | 85                    | 75°     | 82      |
| rSec22b              | 77°                  | 76                    | 68°     | 73      |
| Average              | 87                   | 83                    | 74      |         |

### Table II
Thermal stabilities of different q-SNARE combinations with VAMP2

| VAMP2 | Syntaxin-IA | Syntaxin-4 | Syntaxin-13 | Average |
|-------|-------------|------------|-------------|---------|
| SNAP-23| 79          | 80         | 53          | 71      |
| SNAP-25| 90°         | 86°        | 74°         | 83      |
| SNAP-29| 83°         | 84°        | 70°         | 79      |
| Average| 83          | 84         | 66          |         |

**FIG. 4.** The unfolding transitions for the different q-SNARE combinations. Unfolding transitions are shown for SNAP-23 (A), SNAP-25 (B), and SNAP-29 (C), with SYN1 (circles), SYN4 (squares), and SYN13 (diamonds) complexed with VAMP2.
anterograde and retrograde membrane transport (23). Although the idea that selective pairing of SNAREs mediates vesicle targeting and fusion specificity served as a motivation for characterizing and localizing these proteins, our results suggest that the organization of the secretory pathway relies on other mechanisms to generate specificity.

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