doi:10.1016/j.jmb.2006.09.077 J. Mol. Biol. (2006) 364, 1048–1060

JMB

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Self-interaction of a SNARE Transmembrane Domain Promotes the Hemifusion-to-fusion Transition

Mathias W. Hofmann[1]†, Karolina Peplowska[2,3]†, Jan Rohde[2] Bernhard C. Poschner[1], Christian Ungermann[3] and Dieter Langosch[1]*

[1]*Lehrstuhl Chemie der Biopolymere, Technische Universität München Weihenstephaner Berg 3 85354 Freising Germany*

[2]*Biochemie Zentrum der Universität Heidelberg Im Neuenheimer Feld 328 69120 Heidelberg Germany*

[3]*University of Osnabrück Department of Biology Biochemistry, Barbarastrasse 13 49076 Osnabrück, Germany*

**Corresponding author*

SNARE proteins mediate intracellular fusion of eukaryotic membranes. Some SNAREs have previously been shown to dimerise *via* interaction of their transmembrane domains. However, the functional significance of these interactions had remained unclear. Here, we show that mutating alternate faces of the transmembrane helix of the yeast vacuolar Q-SNARE Vam3p reduces the ability of the full-length protein to induce contents mixing in yeast vacuole fusion to different extents. Examination of liposome fusion induced by synthetic transmembrane domains revealed that inner leaflet mixing is delayed relative to outer leaflet mixing, suggesting that fusion transits through a hemifusion intermediate. Interestingly, one of the mutations impaired inner leaflet mixing in the liposome system. This suggests that the defect seen in vacuolar contents mixing is due to partial arrest of the reaction at hemifusion. Since covalent dimerisation of this mutant recovered wild-type behaviour, homodimerisation of a SNARE transmembrane domain appears to control the transition of a hemifusion intermediate to complete lipid mixing.

Keywords: Vam3p; vacuole fusion; hemifusion; transmembrane domain; SNARE

Introduction

Biological membrane fusion requires the drastic restructuring of lipid bilayers. Lipid mixing is thought to proceed in two sequential steps. In the first step, merger of the contacting, i.e. proximal, monolayers results in an intermediate stage, termed hemifusion. In the second step, merger of the distal monolayers results in full fusion.[1–3] Fusion of most intracellular membranes in eukaryotic cells is driven by SNARE (soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor) proteins. The SNARE family comprises many isoforms that are found on different cellular compartments. Accordingly, different SNAREs are responsible for fusion of synaptic vesicles with the presynaptic plasma membrane, for fusion of yeast secretory vesicles to the cell membrane and for homotypic fusion of yeast vacuoles upon cell division, among others. SNAREs consist of a variable N-terminal domain, a central coiled-coil domain, and, in most cases, of a single C-terminal transmembrane domain (TMD) that is thought to be α-helical.[4] They form stable heterooligomeric complexes upon assembly of their coiled-coil domains.[5] Based on the identity of the amino acid at the central hydrophilic zero-layer of the coiled-coil, SNAREs are classified as Q and R-SNAREs.[6] SNARE complexes may assemble *in cis*, i.e. within the same bilayer, or *in trans*, thus mediating docking of cognate membranes.[3,7,8] Previous studies indicate that *trans* complex formation *via* the cytoplasmic coiled-coil domains is required for fusion, but by itself does not seem to be sufficient for complete bilayer mixing. For example, replacement of the TMDs of yeast exocytotic SNAREs (Snc1p and Sso2p)[9] or of a yeast vacuolar SNARE (Vam3p)[10] by isoprenyl anchors allowed for efficient

† M.W.H. and K.P. contributed equally to this work.

Present address: J. Rohde, German University of Cairo, Al Tagamoa Al Khames, New Cairo City, Egypt.

Abbreviations used: SNARE, soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor; GPI, glycosylphosphatidyinositol; TMD, transmembrane domain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; NBD, 7-nitro-2-1,3-benzoxadiazol-4-yl; Rh, Lissamine Rhodamine B Sulfonyl; TFE, trifluoroethanol; wt, wild-type.

E-mail address of the corresponding author: langosch@lrz.tum.de

trans complex formation, yet abolished full fusion. This indicated a function of the TMD downstream of *trans* complex formation. Recent studies show that yeast vacuole fusion involves a hemifusion intermediate, which follows *trans* complex formation.[11] Other reports relate SNARE TMDs to the transition from hemifusion to complete bilayer mixing. Liposome fusion driven by yeast exocytotic SNAREs is partially arrested in hemifusion if the TMD of the corresponding R-SNARE has been truncated.[4] Moreover, "flipped" synaptic SNAREs, that were expressed with an inverted topology on the surface of eukaryotic cells, induced cell-cell fusion that was completely arrested at hemifusion if the TMDs had been replaced by glycosylphosphatidylinositol (GPI)-anchors.[12] Hemifusion is therefore assumed to be an on-pathway intermediate of SNARE-driven membrane fusion whose completion seems to require proteinaceous TMDs. Moreover, the extent to which SNARE-driven liposome fusion was arrested at hemifusion increased with decreasing density of SNARE proteins in the membrane.[4,13] This is consistent with, but does not necessarily imply, a role of SNARE–SNARE interactions in the hemifusion-to-fusion transition.

Here, we examined the role of a SNARE TMD in membrane fusion. Indeed, TMD–TMD interactions had previously been shown to drive dimerisation of the synaptic R-SNARE synaptobrevin II, of the Q-SNARE syntaxin 1A and of Sso1p.[14–18] Ala-scanning mutagenesis[14] and molecular modelling[19] implied that the synaptobrevin TMD forms a right-handed pair of α-helices that interacts with low affinity.[20] However, the functional significance of these TMD–TMD interactions had remained unclear in these previous studies. Our present results demonstrate that point mutations within the TMD of the vacuolar Q-SNARE Vam3p affect the fusogenic function of the full-length protein in vacuole fusion and of a peptide mimic of its TMD in liposome fusion.

Further, one of the mutants is impaired in its ability to mediate the hemifusion-to-fusion transition. We conclude that this step requires self-interaction of the Vam3p TMD.

Results

To examine the functional relevance of a SNARE TMD, we separately exchanged two sets of four residues within the predicted Vam3p TMD for Ala (Figure 1(a)). The mutated residue patterns are shifted relative to each other by two residue positions and are thus located on alternate surfaces of an α-helical TMD. The functional consequences of these mutations were tested within the context of full-length Vam3p and of a peptide mimic of its TMD.

Vam3p TMD mutations impair vacuole–vacuole fusion

To analyse the effects of the Vam3p TMD mutations in yeast vacuole fusion, we examined

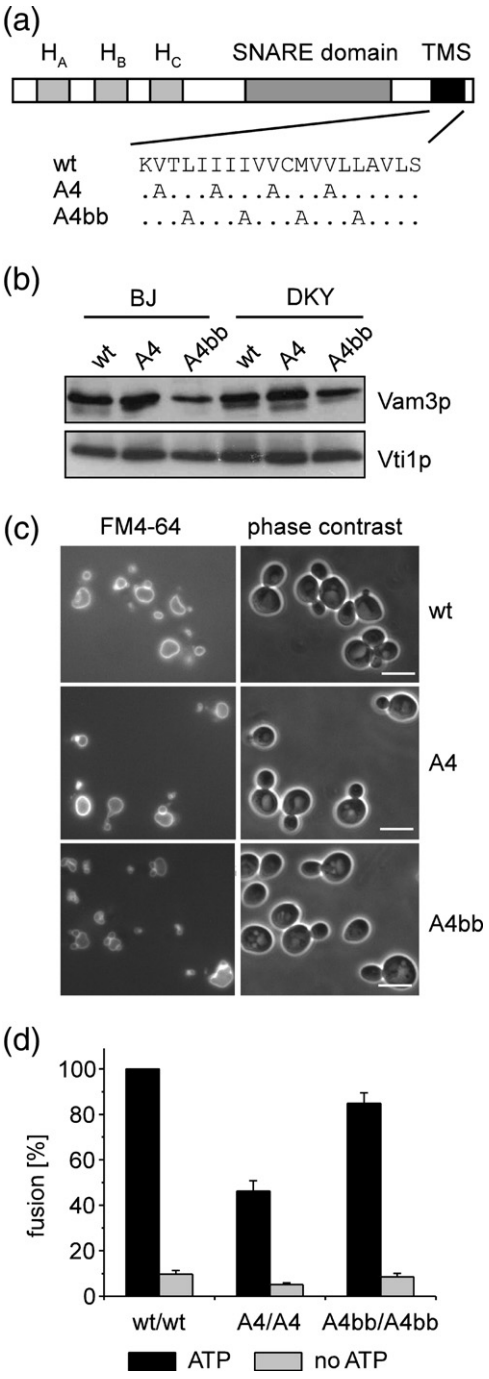

Figure 1. Effects of Vam3p TMD mutations on vacuole morphology and fusion. (a) TMD mutants used in this study. Wt residues of the mutants are represented by dots. (b) Expression level of Vam3p TMD variants. Vacuole protein extracts were analysed by SDS-PAGE and Western blotting using antibodies against Vam3p; for control, the expression of Vti1p was analysed in parallel. (c) Vacuole morphology. Cells (BJ3505) expressing the respective Vam3p variant were stained with FM4-64 and the integrity of the vacuoles was analysed by fluorescence microscopy. Similar results were obtained with DKY6281 cells. (d) *In vitro* vacuole fusion. Purified vacuoles isolated from both fusion tester strains expressing either wt Vam3p or its mutants were mixed and incubated for 90 min at 26 °C in the presence or absence of ATP. Experiments were done in triplicate and contents mixing was determined by a colourimetric assay.