Involvement of LMA1 and GATE-16 family members in intracellular membrane dynamics

Zvulun Elazar *, Ruth Scherz-Shouval, Hagai Shorer

Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 8 January 2003; received in revised form 1 April 2003; accepted 6 May 2003

Abstract

Intracellular membrane fusion is conserved from yeast to man as well as among different intracellular trafficking pathways. This process can be generally divided into several well-defined biochemical reactions. First, an early recognition (or tethering) takes place between donor and acceptor membranes, mediated by ypt/rab GTPases and complexes of tethering factors. Subsequently, a closer association between the two membranes is achieved by a docking process, which involves tight association between membrane proteins termed SNAREs. The formation of such a trans-SNARE complex leads to the final membrane fusion, resulting in an accumulation of cis-SNARE complexes on the acceptor membrane. Thus, multiple rounds of transport and delivery of the donor SNARE back to its original membrane require dissociation of the SNARE complexes. SNARE dissociation, termed priming, is mediated by the AAA ATPase, N-ethylmaleimide-sensitive factor (NSF) and its partner, soluble NSF attachment protein (SNAP), in a reaction that requires ATP hydrolysis. In the present review we focus on LMA1 and GATE-16, two low-molecular-weight proteins, which assist in priming SNARE molecules in the vacuole in yeast and the Golgi complex in mammals, respectively. LMA1 and GATE-16 are suggested to keep the dissociated cis-SNAREs apart from each other, allowing multiple fusion processes to take place. GATE-16 belongs to a novel family of ubiquitin-like proteins conserved from yeast to man. We discuss here the involvement of this family in multiple intracellular trafficking pathways.

© 2003 Elsevier B.V. All rights reserved.

Keywords: Membrane fusion; Golgi; Vacuole; Autophagy; SNARE; NSF; LMA1; GATE-16

1. Introduction

Transport of proteins in eukaryotic cells is mediated by coated vesicles which bud from membrane-bound compartments and are then targeted to and fuse with the appropriate acceptor organelles [1,2]. Based on the current view of vesicular transport, budding is regulated by small GTPases and mediated by cytosolic coat proteins that assemble on the donor membrane [3,4]. Targeting of a transport vesicle to the correct target membrane requires several protein complexes. In some cases, targeting through long distances requires molecular motors and cytoskeleton [5]. The specificity of targeting is mediated by multiple sets of tethering proteins, though the mechanism by which these tethering complexes act is still under investigation (see other reviews in this issue). It appears that different types of transport steps require different sets of tethering complexes. For example, TRAPI (transport protein particle I) is implicated in ER-to-Golgi transport [6,7] whereas TRAPII and COG (conserved oligomeric Golgi complex) are implicated in Golgi retrograde transport [8–10]. The best characterized tethering complex, the exocyst, participates in polarized exocytosis in yeast [11–15] and in mammalian polarized cells [16–18]. Another complex, Class C Vps [also known as homotypic fusion and vacuole sorting (HOPS)], is involved in fusion of a vacuole with vesicles or with other vacuoles [19–21] as well as in Golgi-to-endosome transport [22,23]. The Golgi-associated retrograde protein (GARP) complex has been implicated in the putative endosome-to-Golgi retrograde transport [24,25]. Rab GTPases play a central role in the tethering process, although it appears that tethering complexes such as TRAP and Class C Vps act upstream to Rab activation [19,20,23] whereas COG, GARP and the exocyst function downstream [15,26,27].

In addition to the large tethering complexes described above, tethering in the Golgi and the endosomal fusion systems also involve p115 [28–30] and EEA1 [31], respectively. These large coiled-coil proteins extend from the
vesicle membrane, thereby providing a long-range vesicle capturing machinery. P115 has been implicated in tethering of COPI vesicles with the Golgi [32,33], while Usolp, the p115 homologue in yeast, is essential for tethering of ER-derived vesicles with the Golgi membrane [34]. P115 receptors are two coiled-coil proteins, giantin, a membrane protein on the vesicle [35], and GM130 on the Golgi [36]. GM130 is associated with the Golgi by interacting with GRASP65 [32].

SNARE proteins, considered to constitute the core of this process, mediate the final step in vesicular targeting and fusion. SNAREs are either localized on the target membrane (t-SNARE) or on the vesicle (v-SNARE) [2]. Members of the SNARE family are localized in distinct cellular compartments, and it appears that a different set of v- and t-SNAREs is required for almost every step in membrane trafficking. SNARE complex formation overcomes the energy barrier required for membrane fusion [37,38]. Indeed, using distinct liposomes reconstituted with recombinant SNAREs, v–t-SNARE complex formation has been shown to fulfill the minimal requirement for fusion between the two membranes [39]. The structural regions essential for forming the core of the synaptic SNARE complex have been determined [38,40,41]. The core SNARE complex is a parallel bundle of four coiled α-helices derived from the heptad repeat regions of syntaxin, synaptobrevin and SNAP-25-like t-SNAREs [38,40,41]. Furthermore, using three sets of functionally identified yeast t-SNAREs that mediate the fusion of ER-derived transport vesicles with the Golgi, the homotypic fusion of vacuoles, and fusion with the plasma membrane, it was demonstrated that isolated SNARE proteins encode compartmental specificity and mediate the actual fusion event [42–44]. Hence, specificity of membrane fusion may be achieved through the interaction between tethering factors and SNAREs. Indeed, interaction between some tethering factors and the N-terminal domain of SNARE was reported. The Class C Vps complex functionally binds to the Vam3 N-terminal domain [45], and the GARP complex binds the N-terminal SNARE domain of Tlg1p [26]. Another example of functional interaction between tethering factors and SNAREs is represented by the Sec1 family which keeps the N-terminal of the plasma membrane SNAREs in a closed, inactive conformation [46]. Finally, interaction between EEA1 and syntaxin 13, found in a complex containing also rab5, rabaptin5 and N-ethylmaleimide-sensitive factor (NSF), drives endosomal fusion [31].

Some cases of fusion events are regulated downstream of the SNARE complex assembly [47]. In such cases membrane fusion is initiated by calcium flux which activates calmodulin to bind to V₀, the membranal domain of the vacuolar H⁺-ATPase [47]. This event, in turn, triggers trans-complex formation between V₀ complexes on opposing vacuoles. It appears that this trans V₀ complex contains the vacuolar t-SNARE Vam3p [48]. The actual fusion in this system is triggered by protein phosphatase 1 [49], whose target, however, remains to be identified. A mechanism coupling Sec18p (NSF homologue is yeast) activity and V₀ trans-complex formation has been recently proposed [48]. Accordingly, a set of vacuolar transporter chaperons (Vtc), originally discovered as proteins involved in the delivery of the vacuolar ATPase to the vacuole [50], form a complex with Sec18p, Nyv1p and V₀ [48]. Sec18p activation in this case is involved not only in cis-SNARE disruption but also in activation of V₀. Hence, it was suggested that members of the Vtc family, together with a low-molecular-weight factor 1 (LMA1), are responsible to conserve the SNARE-V₀ activated state [48]. This model further postulates that the release of LMA1 from these complexes is the final step before fusion.

In addition to heterotypic fusion between a transport vesicle and its target membrane, many of the late acting components also participate in homotypic fusion processes between two similar compartments. This process is important in postmitotic vacuole/lysosome- or Golgi-reassembly and in endosomal fusion [51–53]. Hence, multiple rounds of homotypic fusion require unpairing of cis-SNARE complexes. Indeed, factors such as the AAA ATPase, NSF and its assisting factor SNAP (soluble NSF attachment protein), which are involved in SNARE complex unpairing, were first characterized in cell-free systems reconstituting such fusion processes. The dissociation of SNARE complexes prior to tethering and fusion was originally termed ‘priming’ [54], although one should distinguish this priming process from neuronal exocytosis where the term priming refers to all the biochemical reactions that take place between vesicle docking and the actual fusion reaction.

The first part of this review focuses on SNARE priming mediated by NSF, SNAP and two low-molecular-weight proteins, LMA1 and GATE-16, that stabilize the unpaired SNAREs. The second part is devoted to the involvement of the GATE-16 protein family in intracellular membrane trafficking.

2. Vacuole fusion and LMA1

The yeast vacuole goes through a series of cell-cycle controlled budding and fusion events. Shortly after bud emergence, a large vacuole is formed in the daughter cell as a result of homotypic fusion between vacuolar fragments. Utilizing purified vacuoles, cytosolic factors and ATP, Conradt et al. [55] established a cell-free system reconstituting homotypic fusion. This system facilitated the characterization of several factors that stimulate vacuole fusion. Factors such as Sec18p (homologue of NSF) and Sec17p (homologue of SNAP) [56], calmodulin [57], Ypt1p [58], Acylcoenzym A A [56], phosphatidylinositol (4,5) biphosphate [PI(4,5)P2] [49], ergosterol [59] and calcium ions [57]—most of which are known to function in other cell-free systems—were found essential for vacuole fusion. Moreover, this system was also instrumental in the identification of LMA1 [60,61] and protein phosphatase 1 [47] as
fusion stimulators. Recently, the Cdc42p GTPase and actin molecules bound to the vacuole membrane were found to participate in late stages of vacuole fusion [62,63]. The SNARE molecules that participate in vacuole fusion were identified in a search for SNARE homologues located specifically in this organelle, utilizing genomic and cell biology tools. The cell-free fusion assay provided a functional system to analyze their activity. Hence, the t-SNAREs Vam3p, Vti1p, Ykt6p, the SNAP-25-homologue, Vam7p, and the v-SNARE Nyv1p all participate in vacuole fusion [51].

In search for soluble factors that promote vacuole fusion, Xu and Wickner [60] isolated from yeast cytosol two low-molecular-weight factors termed LMA1 and LMA2. It turned out that LMA1 is a complex of thioredoxin and the protease inhibitor IB2, whereas LMA2, which exhibits significantly lower fusion activity, is in fact IB2 [61]. It has been claimed that LMA1 does not act via a redox mechanism since (i) mutating both cysteines on the thioredoxin subunit to serines had no effect on its activity in the fusion assay; (ii) NADPH is not a cofactor for fusion; and (iii) LMA2, which lacks thioredoxin, exhibits low but significant fusion activity. The role of thioredoxin in this process is not clear. Does it act only as a chaperone of IB2, or does it have a more direct role in this system? More detailed structural information, especially on IB2, may help resolve this issue. The closest homologue of IB2 in yeast is p13. Although p13 activity was never determined in vitro, deletion of IB2 or p13 alone has only a mild effect on vacuole morphology, whereas deletion of both genes results in a significant vacuole fragmentation in vivo [54]. In addition to its function in homotypic vacuole fusion, LMA1 also facilitates fusion between ER-derived COPII vesicles and Golgi [34,64], suggesting that it is a general fusion factor.

Based on the kinetics and sequence of events in the vacuolar fusion in vitro, Sec18p and Sec17p act prior to trans-SNARE assembly [65]. This finding is consistent with previous reports demonstrating that NSF and SNAP mediate the dissociation of SNARE complexes [66]. The overall set of reactions responsible for activating vacuoles for subsequent docking and fusion events is termed priming (Fig. 1). Accordingly, Sec18p in its ATP-bound state is associated with cis-SNARE complexes via Sec17p. ATP hydrolysis results in dissociation of the SNARE complex, release of Sec17p and transfer of LMA1 from Sec18p to the unpaired Vam3p [54]. LMA1, in turn, is thought to stabilize the activated, labile Vam3p. Transfer of LMA1 from Sec18p to Vam3p is associated with the release of Sec17p from the vacuolar membrane, suggesting a common binding site for LMA1 and Sec17 on both Sec18p and Vam3p. This reaction requires both PI(4,5)P2 [49] and ergosterol [59].

The systems of priming, tethering and docking interact physically. Hence, tethering factors such as Ypt7p, HOPS and Class C Vps are found in complex with Vam7p [67,68]. Tethering factors regulate nucleotide exchange on rab GTPases and together stimulate the formation of trans-SNARE complexes. Removal of LMA1 from Vam3p, thought to be the last step before fusion, is mediated by the vacuolar transporter chaperone 3 (Vtc3p) [48].

### 3. Golgi fusion and GATE-16

The mammalian Golgi is typically represented as stacks of polarized, flattened cisterneae, alternating with regions rich in vesicles and tubules. The Golgi apparatus receives newly synthesized proteins and lipids from the ER, modifies them as they move along the stacks from cis to trans, and finally sorts them to multiple intracellular and extracellular destinations. Large controversy has evolved concerning the nature of transport through the Golgi stacks. One view is that the cargo is transported via vesicles budding and fusing among stable cisterneae. The other model suggests that the cisterneae themselves move, in a process referred to as cisternal maturation. Recent data led to the evolution of a convergent theory, proposing that anterograde transport is carried out mainly by cisternal maturation, and only partially by
vesicular transport, whereas the main role of COPI-coated vesicles is the retrograde, balancing flow of Golgi- and ER-resident proteins from the maturing cisternae (for some reviews discussing this controversy see Refs. [69–73]).

A variety of cell-free systems that reconstitute distinct transport steps were utilized to study the molecular basis of intracellular transport. A well studied system that reconstitutes transport of proteins between early Golgi cisternae was developed originally by Balch et al. [74]. Several soluble factors acting at late stages of vesicular transport (i.e., tethering, docking and fusion) were isolated as essential factors in this assay. These include NSF, SNAP, p115 and most recently Golgi-associated ATPase enhancer of 16 kDa (GATE-16) [75–79]; the latter is a 117-amino-acid protein, predominantly localized to the Golgi apparatus and found peripherally bound to membranes. P115 was shown to mediate the initial docking (tethering) of COPI vesicles with the Golgi membrane, a step required for the pairing between v- and t-SNAREs [80]. NSF, together with α-SNAP, catalyzes SNARE complex disassembly [66] after a round of fusion via its ATPase activity [65,81,82]. GATE-16 was shown to enhance this activity by its attachment to NSF. NSF/α-SNAP, in turn, stimulate the recruitment of GATE-16 to the unpaired GOS-28 in an ATP-dependent manner [79], ATPase-independent [83] manner. This interaction protects the labile, unpaired GOS-28 from proteolysis.

Recently, it has been shown that the function of GATE-16 as a SNARE protector is essential not only for intra-Golgi transport, but also for the homotypic fusion of post-mitotic Golgi fragments. Muller et al. [83] utilized a cell-free system that imitates the mitotic fragmentation/reassembly of the mammalian Golgi [29,84] to examine the activity of various NSF mutants. One such mutant mimics the comatose mutant of D. melanogaster [85], shown to cause an accumulation of synaptic vesicles and assembled SNARE complexes within synaptic nerve terminals [86,87]. This analysis led to the conclusion that NSF has two separable, sequential functions. The first is the ATPase-dependent SNARE disassembly, which occurs during mitotic Golgi fragmentation, and the second is an evolutionary conserved ATP-dependent ATPase-independent function, required for post-mitotic Golgi reassembly [88]. GATE-16 apparently plays a crucial role in this process, since its interaction with GOS-28 is essential to protect GOS-28 and regulate SNARE function [83]. Thus, it seems that GATE-16 acts as a Golgi-SNARE protector in various physiological membrane fusion events. As described previously, in vacuolar homotypic fusion, Sec18p-catalyzed SNARE disassembly is tightly coupled to the transfer of LMA1 from Sec18p (the yeast homologue of NSF) to the t-SNARE Vam3p. LMA1 maintains Vam3p in an activated form, ready for the next fusion event [54]. The similarity between GATE-16 and LMA1 is striking (Fig. 1). Although there is no clear sequence similarity between GATE-16 and the yeast LMA1, it may very well be that GATE-16 is a mammalian functional analog of LMA1.

GATE-16 shares a high level of sequence identity with an expanding family of proteins that were implicated in various cellular processes associated with intracellular membrane trafficking. Members of this family include light chain 3 (LC3), a subunit of the neuronal microtubule-associated protein complex [89], and gamma-aminobutyric acid receptor type A receptor (GABA<sub>A</sub>R)-associated protein (GABARAP), which was implicated in GABA<sub>A</sub>R trafficking and postsynaptic localization [90–92]. Eight additional loci of GATE-16-related proteins were identified in the Ensemble annotation of the human database. In yeast there is only one known homologue of GATE-16, Aut7/Apg8, which was found to function in membrane dynamics during autophagy [93,94].

Recently, it has been shown that two ubiquitin-like conjugation systems that are essential for autophagy in yeast are conserved in human. GATE-16, LC3 and GABARAP were shown to act as modifiers in one of these systems [95,96]. Conjugation in this system is initiated by HsApg4A, which cleaves GATE-16 downstream of glycine 116 [97], thereby exposing this C-terminal glycine to hApg7. Although several Apg4 mammalian homologues have been recently reported [98], to date only one of the putative HsApg4s has been shown to cleave GATE-16 in vitro [97]. HApg7 acts as an E1 enzyme on GATE-16 (or LC3/GABARAP) [95], transferring it to hApg3, an E2 enzyme [96] that presumably promotes the attachment of GATE-16 to its target. HApg7 conjugates almost equally with each of the GATE-16 homologues, whereas hApg3 conjugates preferentially with LC3. The final target of the GATE-16 conjugation system has not been identified yet. Interestingly, in the yeast system, Aut7p was shown to conjugate not to a protein, but rather to phosphatidylethanolamine (PE) as part of its function in autophagosome formation [99,100]. GATE-16, LC3 and GABARAP were recently shown to be associated with membranes over-expressing hApg3 [95,96,101], suggesting that they too might be conjugated to membranes. The physiological role of this conjugation system is yet unclear. Conjugation to the membrane may facilitate a conformational change(s) in the protein, thereby exposing new sites for interaction. Accordingly, the ability of GABARAP to undergo significant conformational changes in response to increased ionic strength of its environment was recently demonstrated [102]. Alternatively, conjugation of a protein to the lipid bilayer may induce a membranal deformation as part of its role in membrane trafficking. Thus, the first hypothesis assumes that the conjugation serves as a novel mechanism for posttranslational modification of a protein, whereas the second one implies that the conjugation serves to modify the membrane.

The linkage between GATE-16 and ubiquitin is evident not only from the modifier-conjugation system, but also from the crystal structure of GATE-16 [103]. The structure of GATE-16 refined to 1.8 Å shows a ubiquitin fold (the last 90 amino acids) with two additional N-terminal helices...
The recently resolved structures of GABARAP show high similarity to the structure of GATE-16 as discussed later [102,104–106].

Thus far, we have specified five interactors of GATE-16: NSF and GOS-28 in Golgi membrane fusion processes, and HsApg4A, hApg7 and hApg3 in the ubiquitin-like conjugation system. Recently, an additional interactor of GATE-16 has been reported, the Unc-51-like kinase (ULK1), a factor involved in axonal elongation [107]. In addition to its homology to UNC-51, ULK1 shares sequence homology with the yeast Apg1p/Aut3p, a central kinase in autophagy. ULK1 interacts with GATE-16 and GABARAP, and to a lesser extent with LC3. Because ULK1 is involved in the outgrowth of neuronal cells, it is thought that the interaction between ULK1 and GATE-16 is part of the vesicular transport machinery essential for axon elongation [107].

4. Mammalian homologues of GATE-16

4.1. LC3

LC3 is one of the mammalian homologues of GATE-16, exhibiting 38% identity and overall 68% similarity to GATE-16. LC3 was originally identified and co-purified with microtubule-associated protein 1A (MAP1A) and MAP1B from rat brain [89,108]. MAPs are major components of the neuronal cytoskeleton and are assumed to be involved in neuronal morphogenesis. Each MAP is composed of a heavy chain and a light chain. Light chain 1 (LC1) is encoded within the 3’ end of the open reading frame that encodes the MAP1B heavy chain; similarly, LC2 is encoded within the 3’ end of the MAP1A heavy chain. However, LC3, a subunit of both MAP1A and MAP1B, is encoded by a different chromosome than either MAP1A or

![Fig. 2](image-url)
MAP1B. Nonetheless, it is always co-expressed either with MAP1A or MAP1B, though the expression levels of LC3 do not correlate with those of the MAPs [89,108].

Recent studies indicated the involvement of LC3 in autophagy [109–112]. Similar to its yeast homologue Aut7p, LC3 associates with autophagosomal membranes [109]. For this association to occur, LC3 must undergo processing. First, proLC3 (142 amino acids) is cleaved downstream of glycine 120 to form LC3-I, a cytosolic protein detected in SDS-PAGE as 18 kDa. Next, LC3-I undergoes a modification that forms LC3-II, a membrane-associated protein detected as 16 kDa [109].

Notably, it has been reported that the genome of a certain type of cytopathogenic pestivirus contains an insertion sequence which is nearly identical to LC3. The insertion sequence ends at glycine 120. Hence, the cellular processing of LC3 allows the release of a downstream viral polypeptide once the virus infects the host cell [113]. Recently, a similar phenomenon was reported for GATE-16 and GABARAP [114].

As mentioned, LC3 interacts with hApg7 and hApg3, the mammalian homologues of the yeast ubiquitin-like system responsible for the conjugation of Aut7p to membranes [95,96]. Moreover, of the three mammalian homologues of Aut7p, hApg3 conjugates preferentially with LC3 [96]. Combining this data with the processing of LC3 and the observed association with autophagosomal membranes, it appears that LC3 may be the functional analog of Aut7 in mammalian systems. To date, LC3 is the only homologue known to be located onto autophagosomal membranes. GATE-16 and GABARAP might eventually be found to locate onto autophagosomes as well, especially since both share higher sequence similarity with Aut7p than LC3. However, the redundancy that this possibility presents makes it evolutionarily and energetically unfavorable.

4.2. GABARAP

GABARAP was initially discovered in a two-hybrid screen aimed to identify molecules that interact with the M3-M4 loop of the γ2 subunit of the heteromeric pentamer GABA\textsubscript{A}R [90]. GABA\textsubscript{A}R for the neurotransmitter GABA is a ligand-gated chloride channel that mediates inhibitory neurotransmission in the central nervous system. Several studies indicate that this interaction is mediated between amino acids 36–68 of GABARAP and the carboxy-terminal of the intracellular loop [90,102]. Based on its similarity to LC3 and the basic N-terminal region of GABARAP, its putative interaction with tubulin was thoroughly studied. Different reports suggest that GABARAP interacts with both soluble tubulin and microtubules, directing this interaction to the N-terminal region of GABARAP [90,102,115]. Overall, the interactions of GABARAP with both the γ2 subunit of GABA\textsubscript{A}R and tubulin may suggest a role for GABARAP in mediating the interactions between GABA\textsubscript{A}R and the cytoskeleton. Such interactions are presumably needed for correct anchoring and clustering of the receptors in order to assure their density in post-synaptic regions, close to nerve termini that release the GABA neurotransmitters. In accord with this hypothesis, co-transfection of GFP-GABARAP with different GABA\textsubscript{A}R subunits in a quail fibroblasts cell line caused a remarkable increase in the number of clustered receptors, and altered the kinetics of the GABA\textsubscript{A}R activity [116], suggesting an involvement of GABARAP in this process.

Despite these observations, several lines of evidence raised the question of whether GABARAP is directly involved in the receptor clustering mechanism. The fact that GABARAP is widely expressed in many tissues other than neuronal [90,117] suggests involvement in functions other than GABA\textsubscript{A}R clustering/anchoring. Different immunofluorescence studies show that GABARAP is not enriched at synaptic regions, as no significant co-localization was observed between GABARAP and synaptic GABA\textsubscript{A}R, synaptic gephyrin or other pre-synaptic marker proteins [91,118]. Several independent studies show that GABARAP is located mainly in intracellular punctate structures, including the Golgi apparatus and post-synaptic membranes [91,118]. Furthermore, like its close homologue GATE-16, GABARAP interacts directly with NSF [91], supporting direct involvement of GABARAP in intracellular trafficking. The physiological significance of this interaction, however, has still to be elucidated.

As expected from their high sequence homology, GABARAP and GATE-16 share a similar three-dimensional structure. The three-dimensional structure of GABARAP, as determined by NMR spectroscopy and X-ray crystallography [102,104–106], indicates that GABARAP, like GATE-16, possesses a ubiquitin-like core, with an additional N-terminal helical region. Notably, Coyle et al. [102] reported two different conformations for the crystal structure of GABARAP, which diverge significantly only in the first 10-amino-acid region. In the closed conformation structure, the N terminus is embedded in a mini-core hydrophobic pocket found at the interface between the N- and C-terminal domains of the same molecule. In the open conformation, the N terminus is projected away from the ubiquitin-like domain and binds to a hydrophobic pocket of an adjacent molecule found near the S2 strand. The surface of this pocket is composed of highly conserved hydrophobic residues, implying its importance in protein–protein interactions involving all GATE-16 family members. It has been recently proposed that GABARAP may self-associate to form a homodimer in vitro [119]. The reported interaction domain required for this dimerization, amino acids 41–51, is located in the region of this hydrophobic pocket. However, using NMR and other methods, Knight et al. [106] found no evidence for transient oligomerization, suggesting that additional studies are needed to determine whether this dimerization reflects a biological process.

The data obtained to date regarding GABARAP structure and putative functions suggest that this protein is involved
in dynamic interactions with various factors rather than being a major component of the inhibitory post-synaptic scaffold. The intracellular localization of GABARAP and its proposed interaction with GABA\textsubscript{A}R, tubulin and NSF imply that GABARAP may play an important role in membrane trafficking of GABA\textsubscript{A}R-containing transport vesicles. Some of the hypothetical roles that could be fulfilled by GABARAP in this process include sorting of the receptor molecules into budding vesicles, mediating the interactions between transport vesicles and cytoskeleton, or priming of certain SNARE molecules, similarly to GATE-16. These presumed functions of GABARAP could be crucial for efficient and accurate targeting of GABA\textsubscript{A}R to the synaptic regions, or alternatively, from these regions into intracellular organelles such as endosomes or lysosomes. However, whether GABARAP is directly involved in vesicular transport as some of its close homologues remains to be shown.

5. Autophagy and membrane trafficking

Autophagocytosis is a nonselective mechanism for sequestering of cytosolic proteins or organelles into lysosomes and is conserved from yeast to man [120]. It operates constitutively, but can be induced under conditions of stress, in particular nutrient limitation. Two main forms of autophagy are known. Microautophagy, which operates via direct invagination of lysosomes/vacuole, leads to the formation of single membrane-bound vesicles in the lysosomal lumen that are rapidly degraded [120]. This pathway can transfer organelles into lysosomes, as shown extensively for peroxisomes [121–124]. Macroautophagocytosis (hereafter referred to as autophagy), on the other hand, occurs through the formation of autophagosomes, which are specialized vesicles with double or multiple boundary membranes [125,126]. During their formation, these vesicles engulf portions of cytosol or organelles such as mitochondria. The outer autophagosomal membrane fuses with the vacuole to release a single membrane-bound autophagic body into the vacuolar lumen. These autophagic bodies are subsequently degraded in a step that depends on active vacuolar lipases [127] and other hydrolases present in the vacuolar lumen. The process by which autophagosomes are formed is poorly understood. Their origin too remains unclear though the ER, the Golgi complex, and specialized structures called phagophores were proposed as precursors [128–130]. Morphological analysis of yeast in two independent molecular and genetic studies led to the isolation of two classes of mutants defective in autophagy, denoted \textit{apg} and \textit{aut} (both terms stand for autophagy) [131,132], respectively. The \textit{apg} mutants were isolated through their reduced ability to survive during starvation [132], whereas the \textit{aut} mutants were isolated through their defective breakdown of the cytosolic enzyme fatty acid synthase, whose degradation during starvation is dependent on vacuolar proteases [131]. These mutants partially overlap with a third set of mutants, Cvt, cytoplasmic-to-vacuole targeting pathway [133], which were isolated through their defective processing of the vacuolar hydrolase aminopeptidase I (API) [133,134].

At least three membrane fusion events take place along the autophagic pathway. First, small vesicles of unknown origin fuse homotypically with each other to form a double-bilayered membrane termed isolation membrane. Next, the isolation membrane, often found as a typical cup-shaped double-layered membrane, engulfs parts of the cytoplasm and its membrane tips fuse to form a double-membrane autophagosome. This is often referred to as the autophagic sequestration step. Finally, The autophagosomes are targeted to the vacuole and the outer autophagosomal membrane fuses with the vacuole to release a single membrane-bound autophagic body into the vacuolar lumen. Of all these fusion events, only the final fusion between the autophagosome and the vacuole has been thoroughly studied. Vam3p, the vacuolar t-SNARE, Vps18p [21,135] as well as Sec18p and Vti1p [136] were implicated in this step. The mechanism underlying the first two putative fusion events is yet unclear. Of a particular interest is the sequestration of cytoplasm into autophagosomes. It may be regarded as simple homotypic fusion between membrane partners which are already connected, or, as speculated, as a reversal of the Golgi fenestration process [137]. However, the factors involved in such membrane deformations and fusion were not identified yet.

Other membrane trafficking factors were implicated in autophagic processes. In a recent study analyzing the involvement of different secretion factors in autophagocytosis, Ishihara et al. [136] suggested that Sec18p is involved only in the fusion of autophagosomes with the vacuolar membrane. Moreover, factors such as Sec12p, Sec16p, Sec23p and Sec24p, which are known to be involved in the formation of ER-derived COPII vesicles, were found essential for autophagosome biogenesis [136]. Notably, Sec13p and Sec31p, the coat components of the COPII vesicles, do not participate in this process.

The vacuolar protein sorting (\textit{vps}) mutants were identified by screening for strains defective in vacuolar protein transport. Four \textit{vps} gene products (Vps11p, Vps16p, Vps18p and Vps33p) form a hetero-oligomeric protein complex which mediates a late step in protein transport to the vacuole [21]. Analysis of a temperature-sensitive \textit{vps} mutant indicated that, in addition to Vps18p activity in the biosynthetic and the endocytic pathways, its function is required in cytoplasm-to-vacuole protein delivery [138]. At the nonpermissive temperature, \textit{vps18} mutants accumulate various transport intermediates such as multivesicular bodies and autophagosomes [21]. Similarly, a \textit{slp1/vam5/vps16} null mutant accumulates autophagosomes in the cytosol [139]. Additionally, Tcl2p, a member of the syntaxin family of t-SNARE proteins, and Vps45p, a Sec1p homologue, are reported to be required for the constitutive Cvt pathway.
An overlap between endocytosis and autophagy has been demonstrated by the presence of endocytosed material in autophagic vesicles in mammals [141].

5.1. Aut7p

Aut7p is a homologue of GATE-16 in S. cerevisiae, is known to function in membrane dynamics during autophagy [93,94]. Lang et al. [93] first proposed that Aut7p and Aut2p/Apg4p are involved in the delivery of autophagosomes to the vacuole along microtubules. Later, Kirisako et al. [94] showed that Aut7p/Apg8p plays an important role in autophagosome formation. Interestingly, the transcription of Aut7p and some other aut genes is up-regulated under conditions that activate autophagy. It appears that aut7 null strains produce irregular or small autophagosome-like structures whereas normal autophagosomes are absent [94]. Consistently, inhibition of de novo protein synthesis also blocks autophagosome formation while small Cvt vesicles are not affected [142]. It has been therefore suggested that autophagosomal enlargement is associated with an increase in Aut7p levels [142]. Indeed, Aut7p was detected by electron microscopy near regions suspected to represent autophagosome formation zones. Hence, Aut7p may be involved in determining the size of the isolation membrane and subsequently the size and structure of autophagosomes. Consistent with a role in membrane elongation, it was recently demonstrated that Aut7p interacts with Bet1p and Nyv1p, yeast v-SNAREs [143]. It is therefore tempting to propose that Aut7p promotes the homotypic fusion required for enlargement of the autophagic isolation membrane.

In spite of its hydrophilic nature, considerable amounts of Aut7p are covalently bound to membranes, specifically conjugated to PE [99,100]. Recent studies with GFP-Aut7p revealed that it is co-localized with most APG/AUT and CVT gene products in one or a few preautophagosomal structures (PAS) [144–148]. It appears that Aut7p is the first component to be localized onto autophagosomes and Cvt vesicles [94,149]. Aut7p is also unique in being the only characterized protein required for autophagosome formation that remains associated with the membrane, thus serving as the only bona fide marker for autophagosomes. Aut7p is found in three cellular pools: a soluble fraction, peripherally associated with the membrane or covalently conjugated to PE [100]. It is expected that Aut7p first interacts with yet unknown membranal proteins, possibly a SNARE molecule involved at this stage, and only then becomes attached to PE by the conjugation machinery. Localization of Aut7p to the PAS requires PE lipidation as well as two classes of proteins, the Apg12p–Apg5p–Apg16 complex and Apg14p [146]. Apg12p is another ubiquitin-like protein that is conjugated to lysine on Apg5p, thus forming an Apg12p–Apg5p complex essential for autophagy [150]. Apg16p binds to the Apg12p–Apg5p complex to form a higher molecular weight complex which remains localized to the PAS [146,151]. The Apg12p–Apg5p complex is also essential for Aut7p lipidation [146,152]. Despite the fact that these protein complexes are involved in the formation of autophagosomes, the molecular details of this process are still being studied.

The growing body of data summarized above suggests that GATE-16 and its homologues represent a new family of ubiquitin-like proteins involved in membrane dynamics in the context of various physiological processes. The fact that there are several mammalian homologues could imply that each protein utilizes a similar mechanism in different physiological processes. The finding that GATE-16 is a functional analog of LMA1, yet a sequence homologue of Aut7p, is intriguing. Could it suggest that this unique membrane–conjugation system is an evolutionary successful “trick” used by functionally diverse proteins as a mechanism to speed up conjugation/deconjugation from membranes?

6. Concluding remarks

Our understanding of membrane fusion has progressed immensely since the first discovery of SNAREs. These molecules form the general core machinery for intracellular membrane fusion. Many new factors involved in the regulation of this process have been characterized. It appears that a number of chaperone-like molecules are responsible for the formation of a potent trans-SNARE complex in a process that involves significant conformational changes. Hence, SNARE molecules can be found in principle in at least three different conformational states. The first and probably the fusigenic state comprises of a trans-SNARE complex. The second state, formed just after membrane fusion, is the cis-SNAREs complex. The third and probably the most labile state are free SNAREs, formed as a result of a priming reaction mediated by NSF and SNAP. Two transport stimulators, LMA1 and GATE-16, keep the free SNAREs in an active form. Although these proteins share no sequence homology, they appear to function in this process in a similar manner. Both interact with NSF, which concomitantly with SNARE dissociation transfers them to an unpaired free SNARE molecule.

The function of GATE-16 as a SNARE protector was only demonstrated in vitro. In these experimental systems the interaction of GATE-16 with NSF and GOS-28 does not require any additional processing. Clearly, however, GATE-16 and its other mammalian family members are being modified in vivo by hydrolases and conjugases. The target(s) of this novel ubiquitin-like protein family is yet unknown. Additional research is required to determine whether these proteins are also lipidated similarly to Aut7p, their yeast homologue. If so, it is tempting to speculate that such a transient lipidation taking place in the vicinity of primed SNAREs may facilitate membrane fusion.
Acknowledgements

We thank Yigal Avivi for critical reading of the manuscript. Z.E. is incumbent of the Sholimo and Michla Tomarin Career Development Chair of Membrane Physiology. This work was supported in part by the German Israeli Foundation and by The Weizmann Institute Minerva center.

References


Y. Paz, Z. Elazar, D. Fass, Structure of GATE-16, membrane trans-
port modulator and mammalian ortholog of autophagocytosis factor

T. Stangler, L.M. Mayr, D. Willbold, Solution structure of human
GABA(A) receptor-associated protein GABARAP: implications for
13363–13366.

V.N. Bavro, M. Sola, A. Bracher, M. Kneussel, H. Betz, W. Weissenhorn,
Crystal structure of the GABA(A)-receptor-associated protein,

D. Knight, R. Harris, M.S. McAlister, J.P. Phelan, S. Geddes, S.J.
Moss, P.C. Driscoll, N.H. Keep, The X-ray crystal structure and
putative ligand-derived peptide binding properties of gamma-amino-
butyric acid receptor type A receptor-associated protein, J. Biol.

N. Ozakazi, J. Yan, S. Yuasa, T. Ueno, E. Kominami, Y. Masuho, H.
Koga, M. Muramatsu, Interaction of the Unc-51-like kinase and
microtubule-associated protein light chain 3 related proteins in the
brain: possible role of vesicular transport in axonal elongation, Brain

S.S. Mann, J.A. Hammarback, Gene localization and developmental
expression of light chain 3: a common subunit of microtubule-assoc-
iated protein 1A(MAP1A) and MAP1B, J. Neurosci. 21 (1991)
365–46.

D.B. Munafò, M.I. Colombo, A novel assay to study autophagy:
regulation of autophagosome vacuole size by amino acid depriva-

D.B. Munafò, M.I. Colombo, Induction of autophagy causes dramat-
ic changes in the subcellular distribution of GFP-Rab24, Traffic 3

T. Suzuki, M. Nakagawa, A. Yoshikawa, N. Sasagawa, T. Yoshi-
mori, Y. Ohsumi, I. Nishino, S. Ishiura, I. Nonaka, The first molec-
ular evidence that autophagy relates rimmed vacuole formation in

G. Meyers, D. Stoll, M. Gunn, Insertion of a sequence encoding light
chain 3 of microtubule-associated proteins 1A and 1B in a pestivirus
gene: connection with virus cytopathogenicity and induction of

P. Becher, H.J. Thiel, M. Collins, J. Brownlie, M. Orlich, Cellular
sequences in pestivirus genomes encoding gamma-aminobutyric
acid (A) receptor-associated protein and Golgi-associated ATPase

H. Wang, R.W. Olsen, Binding of the GABA(A) receptor-associated
protein (GABARAP) to microtubules and microfilaments suggests
involvement of the cytoskeleton in GABARAP/GABA(A) receptor

L. Chen, H. Wang, S. Vicini, R.W. Olsen, The gamma-aminobutyric
acid type A (GABA) receptor-associated protein (GABARAP) prevents
GABA receptor clustering and modulates the channel kinetics, Proc.

F. Green, T. O’Hare, A. Blackwell, C.A. Enns, Association of
human transferrin receptor with GABARAP, FEBS Lett. 518

M. Kneussel, S. Haverkamp, J.C. Fuhrmann, H. Wang, H. Wassle,
Human GABA(A) receptor subunit intracellular loops: implications
for higher order complex formation, J. Neurochem. 83 (2002)
1164–1171.

P.O. Seglen, P. Bohley, Autophagy and other vacuolar protein de-


S.V. Scott, M. Baba, Y. Ohsumi, D.J. Klionsky, Aminopeptidase I is targeted to the vacuole by a nonclassical vesicular mechanism, J. Cell Biol. 138 (1997) 37–44.


