Fusion of the endoplasmic reticulum by membrane-bound GTPases

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The endoplasmic reticulum (ER) membrane forms an elaborate network of tubules and sheets that is continuously remodeled. This dynamic behavior requires membrane fusion that is mediated by dynamin-like GTPases: the atlastins in metazoans and Sey1p and related proteins in yeast and plants. Crystal structures of the cytosolic domains of these membrane proteins and biochemical experiments can now be integrated into a model that explains many aspects of the molecular mechanism by which these membrane-bound GTPases mediate membrane fusion.

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\textbf{Contents}

1. Introduction
2. Crystal structures of cytosolic domains of the GTPases
3. A comprehensive model for ATL function
4. Futile cycles of ATL
5. The roles of the TM segments and CT
6. Comparison of Sey1p and ATL
7. Connection and comparison with SNARE-mediated membrane fusion
8. Conclusions and perspective
Acknowledgments
References

1. Introduction

Membrane fusion between viral and cellular membranes, as well as between intracellular transport vesicles and target membranes, has been studied extensively. These studies demonstrated that a conformational change of a viral protein or the assembly of SNARE proteins pulls two membranes together so that they can fuse [1–5]. A viral fusion protein generally undergoes the conformational change only once, whereas SNAREs participate in multiple rounds of fusion, as their assembly is counteracted by the dissociation of the complex, a process mediated by the ATPase NSF [6,7].

How endoplasmic reticulum (ER) membranes fuse has been a long-standing question. Light microscopy has shown that the ER forms a dynamic network, in which membrane tubules continuously form and fuse with one another or with edges of membrane sheets [8]. In contrast to viral and SNARE-mediated fusion reactions, the fusing membranes are identical (homotypic, rather than heterotypic fusion). Work over the past seven years has shown that ER fusion is mediated by the atlastins (ATLs) in metazoans, by Sey1p in yeast, and by Sey1p-related proteins in plants (the \textit{Arabidopsis thaliana} homolog is called RHD3 for Root Hair Defective) [9–11].

The ATLs and Sey1p-like proteins are membrane-bound GTPases localized to the ER [9]. They belong to the dynamin family, the founding member of which is dynamin-1, a GTPase involved in the fission of membrane vesicles from the plasma membrane during endocytosis [12]. ATLs and Sey1p-like proteins do not share sequence homology, but they all contain a cytosolic N-terminal GTPase (G) domain, followed by a helical bundle (HB) domain, two closely spaced transmembrane (TM) segments, and a cytosolic C-terminal tail (CT) that includes an amphipathic helix (Fig. 1).
HB domain is significantly longer in the Sey1p proteins. All eukaryotes have either ATL or a Sey1p-related protein, but none appears to have both. Mammals have three isoforms of ATL, with ATL1 being prominently expressed in neuronal cells [13,14]. Mutations in ATL1 can cause hereditary spastic paraplegia, a neurodegenerative disease that is characterized by the shortening of the axons in the longest corticospinal motor neurons in the body [15]. This leads to progressive spasticity and weakness of the lower limbs. Presumably, the lack of ATL fusion activity causes ER morphology defects in the long axons. Neuronal disorders have also been reported for mutations in ATL3 in humans and for deletions of ATL in flies and zebrafish [16–18]. The Sey1p GTPase also has a physiological role, as its absence in Candida albicans reduces the virulence of the organism [19].

A role for GTP hydrolysis in ER fusion was first shown by the GTP-dependent “coalescence” of ribosome-stripped rough microsomes [20]. Later experiments used a visual assay to demonstrate that the formation of a tubular ER network from a small Xenopus laevis egg membrane vesicles is dependent on GTP hydrolysis [21]. However, the identity of the GTPase remained unclear. Several reports suggested that Rab GTPases might be involved [22–24], but these studies did not agree on the specific Rab protein involved. Fusion of the Xenopus membranes could be reduced by extracting Rab proteins with GDI [22,23,25], but re-addition of Rab proteins was not reported, leaving the possibility that GDI may have had a nonspecific effect. As we discuss below, it is now likely that the GTP requirement in Xenopus egg extracts is not due to Rab proteins. Whether Rabs have a role in other cells requires further investigation.

A role for ATL in ER fusion was initially shown in Drosophila melanogaster and tissue culture cells [9,26]. The depletion or conditional inactivation of ATL led to long, unbranched tubules or fragmented ER. Non-branched ER tubules were also observed when dominant-negative ATL mutants were expressed [9,14], which presumably form inactive complexes with the endogenous ATL. In addition, the fusion of ER vesicles in Xenopus laevis egg extracts was prevented by the addition of an ATL antibody or a cytosolic fragment of ATL [9,27]. A cytosolic fragment carrying a point mutation that prevented the interaction with the endogenous ATL protein was unable to block ER fusion [27]. Thus, in the Xenopus system, most ER fusion appears to be mediated by ATL. The most convincing evidence for ATL being a fusogen came from the observation that proteoliposomes containing purified Drosophila ATL undergo GTP-dependent fusion in vitro [26,28]. GTP-competent ATL molecules had to be present on both membranes to allow their fusion [26,28].

A role for Sey1p in ER fusion in Saccharomyces cerevisiae was initially based on (1) the similarity to ATL in its GTP-binding motifs and membrane topology, (2) its genetic interaction with an ER tubule-shaping protein (Yop1p), and (3) on ER morphology changes observed in deletion mutants [9]. Additional experiments showed that ER fusion is delayed in SEY1-deletion mutants [10], that ATL and Sey1p can replace each other in yeast and mammalian cells [10,29], and that reconstituted Sey1p-containing proteoliposomes undergo GTP-dependent fusion [10,29], similar to those containing ATL. Deletion of the Sey1p-related RHD3 protein in Arabidopsis causes the shortening of root hairs and ER morphology defects resembling those of ATL depletion in mammalian systems [30,31]. Finally, RHD3 can replace Sey1p in yeast, and reconstituted purified RHD3 mediates GTP-dependent membrane fusion in vitro [11]. All these experiments indicate that Sey1p and its relatives are functional orthologs of ATL.

2. Crystal structures of cytosolic domains of the GTPases

Structural studies of the cytosolic domains of ATL have sought to explain GTP hydrolysis–dependent membrane fusion. Initially, two different conformations of the cytosolic domain of human ATL1 were reported (Fig. 2). In both structures, ATL forms a dimer with the G domains facing one another [28,32]. The nucleotides are bound at the interface between the G domains. In one of the structures, called crystal form 1, the following HB domains cross and then run parallel to one another. The C-termini are only ~10 Å apart, suggesting that the following TM segments in the two full-length proteins would sit in the same membrane. In the other structure, called crystal form 2, the G domains are arranged in a similar way as in crystal form 1, but the two HB domains point in different directions. The corresponding full-length proteins would thus localize to different membranes and tether them together. Based on these considerations, it was proposed that crystal forms 2 and 1 correspond to pre- and post-fusion states, respectively; the conformational change would bring the membranes close together for fusion. However, both structures contained only bound GDP, even though crystal form 2 was obtained in the presence of GDP plus Pi. It therefore remained uncertain whether the conformational change was connected to the GTPase cycle. Biochemical experiments demonstrated that ATL forms much stronger dimers in the presence of GTP than GDP [28], suggesting that the high protein concentration during crystallization favored dimerization in the GDP-bound state.

Another human ATL1 structure (crystal form 3) was later obtained in the presence of GMPPNP or GDP plus AlF4−, conditions corresponding to a state before Pi release in the GTPase cycle [33]. However, this structure only caused more confusion over how conformational changes of ATL induce fusion, as it resembled the post-fusion state, rather than the expected pre-fusion state. In fact, the HB domains of the two ATL molecules were even closer to one another than in crystal form 1. Similarly puzzling was a structure of GDP-bound Drosophila ATL [34], which had a pre-fusion-like conformation. In summary, the crystal structures did not lead to a
coherent picture of how the GTPase cycle of ATL relates to conformational changes.

Because of these confusing results, it is not surprising that conflicting models have been proposed for ATL-mediated membrane fusion. In one model, GTP hydrolysis would happen after fusion [35], but this is difficult to reconcile with the observation that fusion in vitro requires GTP hydrolysis. A second model posits that GTP hydrolysis occurs in ATL monomers sitting in different membranes [33], which would subsequently form a dimer (trans-dimer). Because there is little dimerization in the presence of GDP, the interaction would have to occur between ATL molecules that are in the short-lived transition state of GTP hydrolysis. Not only is this state poorly populated, but it also forms dimers very slowly [33,36]. It has also been proposed that ATL molecules interact only in the same membrane (cis-interaction), rather than in trans across the two apposed membranes; the cis-dimers would induce high membrane curvature in both lipid bilayers, which would then induce their fusion [37]. This model implies that there is no membrane-tethering step involving ATL, which is contradicted by experiments demonstrating ATL-mediated vesicle tethering using several different techniques [36,38].

3. A comprehensive model for ATL function

An important clue for the reconciliation of the crystal structures with a fusion mechanism came from experiments showing that nonhydrolyzable GTP analogs or the presence of GDP and AlF\textsubscript{4}\textsuperscript{−}, which induces a transition state during GTP hydrolysis, cause dimerization of the cytosolic domain of ATL, but full cycles of GTP hydrolysis are required for membrane tethering [33,36,38]. This suggested the possibility that dimers of ATL form on the same membrane (cis-interaction) and compete with the trans-dimers that mediate tethering [36]. This idea was tested by experiments in which ATL molecules were attached to a supported bilayer, so that they could only interact in cis and not with ATL molecules sitting in a different membrane (trans-interactions) [36]. Indeed, a nucleotide-dependent interaction of ATL molecules was observed. These cis-interactions were reduced when a cytosolic ATL fragment was added to the bulk solution, suggesting that trans- and cis-interactions can compete with one another. The existence of cis-interactions explains the puzzling observation that the presence of GDP and AlF\textsubscript{4}\textsuperscript{−} leads to the formation of tight ATL dimers, but does not allow vesicle tethering or fusion [36]. The cis-dimers formed under these conditions cannot dissociate, leaving no monomers for trans-interactions. GTP hydrolysis is thus required to regenerate a pool of monomeric ATL molecules in each membrane, which can then mediate the tethering and fusion of opposing membranes.

The existence of both cis- and trans-dimers can explain the crystal structures. Crystal form 3 corresponds to the transition state conformation formed during both cis- and trans-interactions. Crystal form 2 corresponds to a state following GTP binding. However, the GTP-bound state can probably adopt several different conformations, because GTP binding causes the G domains to undock from the HBs [33], allowing the two domains to move relative to one another. Although crystal form 2 was initially thought to form only in trans, the two ATL molecules may actually sit in the same membrane, with the HBs nearly parallel to the plane of the membrane (Fig. 2). Finally, crystal form 1 probably represents one of the intermediate states that the ATL dimer can adopt during the transition from a conformation corresponding to crystal form 2 to one corresponding to crystal form 3.

A plausible model for ATL-mediated membrane fusion begins with GTP binding to ATL monomers (Fig. 3). ATL likely needs to be in a conformation corresponding to crystal form 2, because GTP loading requires the first helix of the HB to interact with the base of the G domain [33]. In the next step, the monomers rapidly form a dimer, with the bound GTP molecules buried at the interface. This process is likely reversible (not shown in Fig. 3). The dimer is relatively unstable and can adopt different conformations once the G domains are released from the HBs. When GTP is hydrolyzed by the dimer, the two HBs associate with one another, generating a tight dimer in the transition state. Finally, P\textsubscript{i} and GDP are released in a sequen-
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Fig. 3. A model of ATL function. The scheme indicates different stages during the GTPase cycle of ATL. Stage A: ground state, in which ATL molecules (in red and yellow) are in the nucleotide-free, monomeric state. Stage B: ATL molecules bind GTP and form dimers either in the same membrane (cis-interaction; red–red) or across different membranes (trans-interaction; red–yellow). Stage C: following GTP-binding, the G-domains of ATL are dislodged from the HB-domains and can rotate (indicated by the arrows). Stage D: ATL is in the transition state of GTP hydrolysis (GDP–Pi), in which the HBs of the two molecules interact tightly with one another. These trans-interaction lead to membrane fusion. Stage E: ATL molecules have released Pi, and are in the GDP-bound state. Dissociation of the dimer may occur, but is probably completed after GDP-release, i.e., transition back to stage A. Stages F and G: trans-interaction of ATL molecules leads to tethering, but not fusion. This futile tethering cycle (purple arrows) is completed when ATL converts back to the original state (stage A). Note that cis-interacting ATL molecules also undergo a nucleotide-dependent futile interaction that does not lead to membrane fusion.

tial manner, causing the dissociation of the dimer into monomers. It remains unclear whether dissociation occurs in the GDP-bound state, in which the monomers have a weak affinity for each other, or in the nucleotide-free state, in which they have no measurable affinity [36]. All reactions can occur with ATL molecules sitting in different or the same membranes (trans- and cis-interactions, respectively), but membrane tethering and fusion can only occur with trans-interactions. The tight interaction of the monomers in the transition state of GTP hydrolysis likely pulls the two membranes together so that they can fuse.

4. Futile cycles of ATL

ATL not only undergoes an apparent futile GTPase cycle involving the formation and dissociation of cis-dimers. It also forms trans-dimers in a futile manner. Experiments with reconstituted proteoliposomes showed that multiple GTPase cycles are required for a successful fusion event [36,38]: the transition state of trans-dimers often converts back into monomers without having caused fusion. Other experiments indicated that the efficiency of tethering and fusion increases with the density of ATL molecules in the two membranes [36]. Taken together, it seems that a successful fusion event requires the cooperation of multiple trans-dimerization events: as one dimer reaches the transition state, other dimers forming nearby would help to maintain the tethered state and add to the pulling force exerted on the opposing membranes. The futile formation of trans dimers suggests that a fraction of three-way junctions in cells may actually consist of tethered, rather than fused, tubules.

The futile assembly and disassembly of cis-dimers may simply be a consequence of using a single protein to mediate multiple rounds of membrane fusion. ATL molecules have to cycle between being in the same and different membranes, and during the actual fusion process they must interact with one another in a conformation that is close to either of these situations. Thus, one would expect the formation of at least some cis-dimers, which would have
to dissociate to regenerate monomers. cis-dimers may generally be formed at the site of fusion both before and after trans-dimers caused bilayer merging. Multiple futile cycles of trans-dimerization that lead to tethering may cause the localized enrichment of monomers that could then engage in cis-dimerization in each membrane. cis-dimerization at the site of membrane tethering would effectively lower the energy barrier for the fusion event. cis-dimers might also form after fusion and transiently keep ATL and Sey1p in newly formed three-way junctions, which might explain the enrichment of some isomers in junctions [23,29]. Whether such cis-dimers stabilize fused three-way junctions [39] remains to be tested.

5. The roles of the TM segments and CT

The TM segments of ATL are not mere membrane-anchors. They cannot be replaced by the TM domains from other proteins, and some point mutations in the TMs severely affect the efficiency of fusion [40]. Thus, the TMs might contribute to the formation of a fusion intermediate, such as a hemifusion stalk or fusion pore. In addition, they mediate the formation of nucleotide-independent ATL oligomers, which could increase the local concentration of ATL molecules at the site of fusion, thus increasing the fusion efficiency. Finally, the hairpin structure formed by the closely spaced TMs probably targets ATL to high-membrane curvature regions of the ER, such as tubules and sheet edges, as similar hairpin structures are found in other proteins localized to these regions [41,42].

The C-terminal tail (CT) contains an amphipathic helix that promotes membrane fusion [40,43]. ATL lacking the CT has only low fusion activity, but it can be re-activated by addition of a synthetic peptide corresponding to the amphipathic helix [40]. The helix acts by directly binding to and perturbing the phospholipid bilayer, which could facilitate the merging of the two membranes during fusion. Binding of the helix to the membrane probably occurs at all stages of the fusion process. Phosphorylation of the CT of RH3D has been reported to stimulate tubule formation, but this modification seems to be confined to this particular GTPase [44].

6. Comparison of Sey1p and ATL

Recent experiments show that Sey1p functions similarly to ATL [29]. Crystal structures of the cytosolic domain of C. albicans Sey1p show that it forms a dimer in GMPNP or GDP plus AIF₂, and a monomer in GDP. In the dimeric state, the HB segments of the two Sey1p molecules crossover twice, such that the C-termini are on the same side as the G domains (Fig. 2). Surprisingly, when the reconstituted full-length proteins were tested in a fusion assay, some lipid mixing was observed even with the non-hydrolyzable GTP analog GMPNP, although fusion was much more efficient with GTP and was not observed with GTPS [10]. It is possible that Sey1p monomers form the tightest dimer in their GTP-bound state, rather than in the transition state as ATL monomers do.

7. Connection and comparison with SNARE-mediated membrane fusion

In Xenopus extracts and Drosophila cells, most if not all ER fusion seems to be mediated by ATL, as its inhibition or absence causes the ER to fragment [26,27]. Similarly, the Sey1p-homolog RHD3 may be the only fusogen in A. thaliana, as its deletion causes drastic ER morphology defects [31]. In contrast, the deletion of SEY1 in S. cerevisiae does not grossly disturb the cortical ER network [9], and membrane fusion is delayed, but not abolished [10]. The alternative fusion pathway in S. cerevisiae is likely mediated by ER SNAREs. Mutants of the ER SNAREs Ufe1p, Sec22p, or Use1p have a drastic effect on ER morphology when combined with a deletion of SEY1 [10]. In addition, combining a temperature-sensitive mutant in Ufe1p with a SEY1 deletion results in a much slower fusion rate than with each mutant individually [10]. The SNARE-mediated ER fusion process also requires the tethering protein Dsl and is distinct from that of Golgi-derived vesicles with the ER [45]. These data suggest that Sey1p and ER SNAREs function in alternative fusion pathways in S. cerevisiae, although it is unclear whether both pathways operate in parallel in normal cells.

A different model has been proposed in which Sey1p and ER SNAREs function together in the same pathway [46]. The authors suggested that the Sey1p GTPase causes only the tethering of membranes, like the Rab GTPases, whereas the SNAREs and NSF ATPase perform actual fusion. However, this model is difficult to reconcile with the fact that Sey1p alone can mediate membrane fusion [10,29], and that double mutants in SEY1 and SNAREs have a much stronger effect on fusion or cell growth than the individual SEY1 or SNARE mutants [10]. These results strongly argue that these proteins act in alternative pathways, rather than the same.

It is interesting to compare ATL/Sey1p-mediated membrane fusion with that involving the SNAREs. In both cases, nucleotide hydrolysis is required, but for ATL/Sey1p this energy is spent during the actual fusion reaction, whereas for SNAREs it is used for the recycling of the complex after fusion. In both systems, cis- and trans-complexes form, but in the case of SNAREs, it is uncertain whether they compete. SNAREs interact with several factors, including SM proteins, which could prevent cis-assembly and catalyze the formation of trans-complexes [47]. In contrast, no factors have been identified that affect the fusion activity of ATL or Sey1p-like proteins. Fufit tethering cycles may occur for SNAREs as well, as trans-complexes were observed to disassemble before they caused fusion [48]. As in the case of ATL, fusion is more efficient when multiple SNARE complexes assemble simultaneously [49]. Thus, in both systems, not every event of trans-interaction seems to result in fusion.

8. Conclusions and perspective

Over the past years, significant progress has been made in our understanding of the mechanism by which ATL and Sey1p mediate the fusion of ER membranes. Because fusion is mediated by identical molecules in both membranes and because no other proteins seem to be involved, the reaction is significantly simpler than that mediated by SNAREs. In addition, crystal structures are available and the fusion reaction can be recapitulated with purified proteins in reconstituted proteoliposomes, triggered simply by the addition of GTP. These features make membrane-bound GTPases particularly attractive to analyze the molecular mechanism of membrane fusion. Indeed, the combination of structural information and biochemical data has led to a comprehensive model of ATL-mediated fusion [Fig. 3]. Perhaps the most surprising aspect of this model is the existence of two types of futile cycles. Several aspects of the fusion mechanism may also apply to the related GTPases that fuse the membranes of mitochondria. Interestingly, mitofusin (called Fzo1p in yeast), which mediates outer membrane fusion, only forms dimers when actively hydrolyzing GTP [50], suggesting that it also undergoes futile cycling.

Despite the recent progress, we do not understand how exactly ATL and Sey1p catalyze the merging of phospholipid bilayers. Elucidation of the actual fusion mechanism will require the generation of intermediates and the determination of the membrane structure in each of them. The role of lipids needs to be studied in more detail, as these likely play an important role, demonstrated by observations with SNARE-mediated fusion [51] and preliminary results on Sey1p [52]. Future studies will likely require the use of biophys-
methodical to understand the details of the actual fusion process, and structural characterization of full-length ATL or Sey1p. Understanding the precise configurations of the TMs and CT domains that interact with lipids during fusion, will be crucial to determining how fusion is catalyzed. Comparison of the membrane domains of SNAREs [53] and ATLs could give new insights into how a completely different set of protein domains evolved to perform the same task. The biological function of ATL and Sey1p-like proteins is not only entirely understood. While it is clear that these proteins mediate ER fusion, it seems possible that they have additional functions, such as in stabilizing newly formed tubular junctions. ATL and Sey1p-like proteins also interact through their TM segments with other membrane proteins that determine ER morphology. Such interactions have been reported for the reticulons and DP1 (Yop1p in yeast) [9], which are implicated in generating and stabilizing ER tubules, and for the lunapark protein [54], which might stabilize tubular junctions [39,55]. However, how exactly ATL and Sey1p-like proteins cooperate with these proteins is unknown, and it is unclear what specific roles these components play in generating the tubular ER network. Thus, probably the most important goal in the field is to reconstitute a tubular membrane network with purified proteins, a system that would allow in-depth studies on the relationship between membrane fusion and -shaping and on the molecular mechanism by which ER morphology is achieved.

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