The Atlastin amphipathic tail destabilizes membrane structure

Joseph E. Faust1,4, Tanvi Desai1,4, Avani Verma1,4, Idil Ulengin2, Tzu-Lin Sun3, Tyler J. Moss1, Miguel A. Betancourt1, Huey W. Huang3, Tina Lee2, and James A. McNew1

1From the Department of Biochemistry and Cell Biology, Rice University, Houston TX 77005
2Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213
3Department of Physics and Astronomy, Rice University, Houston TX 77005
4These authors contributed equally to this work

*Running title: The Atlastin amphipathic tail destabilizes membrane structure

To whom correspondence should be addressed: James A. McNew, Department of Biochemistry and Cell Biology, Rice University, 6100 Main Street Rice University, MS601, Houston TX 77005 USA, Tel.:713-348-3133, E-mail: mcnew@rice.edu

Keywords: cell compartmentalization; endoplasmic reticulum (ER); membrane fusion; fluorescence resonance energy transfer (FRET); phospholipid vesicle; neurodegeneration; membrane structure; GTPase

Background: Atlastin is a large GTPase that catalyzes the homotypic fusion of ER membranes.

Results: In vitro and in vivo studies reveal that the C-terminal tail of Atlastin affects its function.

Conclusion: The amphipathic C-terminal tail of Atlastin destabilizes lipid bilayers to promote membrane fusion.

Significance: Describing the mechanism of Atlastin-mediated fusion is a critical step in our understanding of ER structure formation.

ABSTRACT

Fusion of tubular membranes is required to form three-way junctions found in reticular subdomains of the endoplasmic reticulum (ER). The large GTPase Atlastin has recently been shown to drive ER membrane fusion and three-way junction formation. The mechanism of Atlastin-mediated membrane fusion is distinct from SNARE-mediated and many details remain unclear. In particular, the role of the amphipathic C-terminal tail of Atlastin is still unknown. We have found that a peptide corresponding to the Atlastin C-terminal tail binds to membranes as a parallel alpha helix, induces bilayer thinning, and increases acyl chain disorder. The function of the C-terminal tail is conserved in human Atlastin. Mutations in the C-terminal tail decrease fusion activity in vitro, but not GTPase activity, and impair Atlastin function in vivo. In the context of unstable lipid bilayers, the requirement for the C-terminal tail is abrogated. These data suggest that the C-terminal tail of Atlastin locally destabilizes bilayers to facilitate membrane fusion.

The structure and function of intracellular organelles, particularly those in the secretory pathway, rely heavily on membrane and content exchange. In most cases, this exchange is accomplished by regulated membrane fusion. Cargo transport throughout the secretory pathway depends on specific targeting and fusion of the vesicular intermediates by SNARE proteins and their associated regulatory factors. The mechanism by which SNARE proteins drive membrane fusion is fairly well-established (1,2), however; membrane fusion events within the endomembrane system are still poorly characterized (3).

The morphology and function of the endoplasmic reticulum is inextricably linked to architectural shape changes. The interconnected network of ER tubules and sheets makes physical contact with virtually every cytoplasmic organelle (3). The dynamic nature of ER tubules allows for rapid shape changes, which likely correspond to
differences in functional interaction (4,5). One of the major players involved in ER shape is the membrane fusion protein Atlastin (6). We and others have shown that the GTPase Atlastin drives the fusion of ER tubules and this fusion reaction is vitally important for overall function of this organelle (6,7) Atlastin interacts with a variety of other proteins resident within the ER, like the ER shape forming proteins reticulon, REEP, and DP1 (7), spastin (8,9) and luna park (10).

The mechanism by which Atlastin fuses ER membranes is beginning to be elucidated. We know that membrane fusion requires GTPase activity (6,7), oligomerization (11), and structural elements within the extreme carboxy terminus of the protein (11,12). In this work we examine the requirements and characteristics of the C-terminal juxtamembrane amphipathic alpha helix. We found that a peptide corresponding to 23 amino acids immediately after the second transmembrane domain forms a helix in the presence of membranes and directly interacts with membrane surfaces. We also show that membrane interaction alters the physical properties of the bilayer such as membrane thinning and disorganization of fatty acid acyl chains. Targeted point mutations within the hydrophobic face of the amphipathic helix resulted in reduced membrane fusion in vitro consistent with a role for amphipathic destabilization during the membrane fusion cycle. Additionally, we found that Drosophila Atlastin functions in mammalian cells where it also requires its C-terminal tail. Somewhat surprisingly, we found that mutations within the C-terminal helix were much more tolerated in vivo than in vitro. This result is likely due to alterations in the lipid composition of native membranes versus synthetic membranes used in vitro fusion. We tested this directly by altering the lipid composition of synthetic proteoliposomes and found that phosphatidylethanolamine, which promotes non-bilayer forming structures, can help alleviate the requirement for the C-terminal tail. In total, these results establish that the Atlastin C-terminal tail is a membrane perturbing alpha helix that participates in membrane fusion by destabilizing bilayer structure and promoting the mechanics of lipid mixing.

**EXPERIMENTAL PROCEDURES**
The Atlastin amphipathic tail destabilizes membrane structure

pJM866 (GST- dAtl(Human Atl-1 C-terminal tail)-His8): The Hs-Atl1 C-terminal tail was generated by PCR using primers BspEI-Hsatl1-c-tail (ATTCCCGGAGAATACCGAGAGCTGG) and Hs_Atl_XhoI (TTCTCGAGCATTTTTTTCTTTTC). The PCR product was digested with BspEI and XhoI ligated into pJM681 cut with the same enzymes.

pJM868 (GST- dAtl(Human Atl-3 C-terminal tail)-His8): The Hs-Atl3 C-terminal tail was generated by PCR using primers BspEI-Hsatl3-c-tail (ATTCCGGACAATATCGTGAGCTGGGC) and Hs_Atl_XhoI (TTCTCGAGCATTTTTTTCTTTTC). The PCR product was digested with BspEI and XhoI ligated into pJM681 cut with the same enzymes.

pJM1082 (GST-dAtl(L482K,A486D,L489D,F493D)-His8): A gene fragment corresponding to residues 461 to 541 of dAtl, containing the L482K, A486D, L489D, and F493D point mutations, was synthesized by IDT, cut with SacI and XhoI, and ligated into pJM681 cut with the same enzymes.

pJM1084 (GST-dAtl(G480K)-His8): A gene fragment corresponding to residues 461 to 541 of dAtl, containing the G480K point mutation, was synthesized by IDT, cut with SacI and XhoI, and ligated into pJM681 cut with the same enzymes.

pJM850 (Venus-dAtl): Venus was PCR amplified from Venus-GRX2 in pET28a (gift from Joff Silberg) using the oligos KpnI Venus A (CGGGTACCATGGTGAAG) and Venus-BamHI 3’ (TAGGATCCGGCAGCGCTTGTA), cut with KpnI and BamHI and ligated into pcDNA3.1 cut with the same enzymes. dAtl was PCR amplified from pJM681 using the oligos BamHI-Dm_Atl (CCGGATCCATGGCGGATCGGC) and Dm_Atl-XhoI (ATCTCGAGTCATGACCGCTTCACC), cut with BamHI and XhoI, and ligated into pJM850 cut with the same enzymes.

pJM1004 (Venus-dAtl(L482K)): dAtl(L482K) was PCR amplified from pJM882 using the oligos BamHI-Dm_Atl (CCGGATCCATGGCGGATCGGC) and Dm_Atl-XhoI (ATCTCGAGTCATGACCGCTTCACC), cut with BamHI and XhoI, and ligated into pJM850 cut with the same enzymes.

pJM1005 (Venus-dAtl(K51A)): dAtl(K51A) was PCR amplified from pJM694 (GST-DmAtlastin (K51A) His8) using the oligos BamHI-Dm_Atl (CCGGATCCATGGCGGATCGGC) and Dm_Atl-XhoI (ATCTCGAGTCATGACCGCTTCACC), cut with BamHI and XhoI, and ligated into pJM850 cut with the same enzymes.

The following mutants were generated using QuikChange site-directed mutagenesis PCR (QIAGEN) and fully verified by sequencing. Venus-dAtl (A486D): The point mutation A486D was generated using quickchange primers dAtl A486D_FWD (GGCAAGTTGGATGACTTTGATACGCTATT GTGGGAG) and dAtl A486D_RVS (CTC CCA AATAGCGTATCAAAGTCATCCAACTTGCC) with pJM850 as a template.

Venus-dAtl (L482K;A486D;L489D;F493D): The quadruple mutant was generated using quickchange primers dAtl _L482K_A486D_L489D forward (GGGTCGCATGAATTTCTCCAATCTACGGATCAACTTGCC) and dAtl _L482K_A486D_L489D reverse (GGGTCGCATGAATTTCTCCAATCTACGGATCAACTTGCC) with pJM850 as a template.

Venus-dAtl (L482K;A486D;L489D;F493D): The quadruple mutant was generated using quickchange primers dAtl _L482K_A486D_L489D_F493D forward (GGGTCGCATGAATTTCTCCAATCTACGGATCAACTTGCC) and dAtl _L482K_A486D_L489D_F493D reverse (GGGTCGCATGAATTTCTCCAATCTACGGATCAACTTGCC) with pJM850 as a template.

Venus-dAtl (L482K;A486D;L489D;F493D): The quadruple mutant was generated using quickchange primers dAtl _L482K_A486D_L489D_F493D forward (GGGTCGCATGAATTTCTCCAATCTACGGATCAACTTGCC) and dAtl _L482K_A486D_L489D_F493D reverse (GGGTCGCATGAATTTCTCCAATCTACGGATCAACTTGCC) with pJM850 as a template.

Pep tide Production— Two peptides, RYSgelsDFFGKLDdFatllWeK (C23) and RYSGELSDFGKLDdFatllWeK (C23-KK), corresponding to the C-terminal tail of Drosophila Atlastin were purchased from GenScript. The
peptides contained an N-terminal acetylation and a C-terminal amidation.

**LUV preparation, circular dichroism, and X-ray Diffraction** – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) were purchased from Avanti Polar Lipids (Alabaster, AL). POPC and DOPS were mixed in a 85:15 molar ratio. Dried lipids were resuspended in Tris buffer solution (10mM, pH 7) to 1 mg/ml. This stock was fully mixing and exposed to more than 5 freeze-thaw cycles. A mini-extruder from Avanti Polar Lipids and Whatman Nuclepore Track-Etch 0.2 µm filters from GE Healthcare were used to produce liposomes. CD, OCD, and X-ray diffraction experiments were performed as previously described (14,15).

**Proteoliposome Production** – Proteins purified in 0.1% Anapoe X-100 (Anatrace) were reconstituted into preformed 100 nm liposomes as previously described (6). Liposomes were made by extrusion. Lipid mixes used were (1) Unlabeled (POPC) and labeled lipid mixes (POPC:Rhodamine-DPPE: NBD-DPPE, 97:1:1.5 mole ratio) (2) Unlabeled lipid mixes (POPC:DOPS; 85:15 molar ratio) and labeled lipid mixes (POPC:DOPS:Rh-DPPE:NBD-DPPE; 82:15:1.5:1.5 mole ratio) (3) Unlabeled lipid mixes (POPC:DOPS:DOPE; 75:15:10 molar ratio) and labeled lipid mixes (POPC:DOPS:DOPE:Rh-DPPE:NBD-DPPE; 75:15:7:1.5:1.5 mole ratio) (4) Unlabeled lipid mixes (POPC:DOPS:DOPE; 65:15:20 molar ratio) and labeled lipid mixes (POPC:DOPS:DOPE:Rh-DPPE:NBD-DPPE; 65:15:17:1.5:1.5 mole ratio). All the lipid mixes had trace amount of [3H]-1-palmitoyl 2- palmitoylphosphatidylethanolamine (DPPE) in chloroform were dried under a stream of N₂ gas followed by further drying in a vacuum for 30 min. The lipid films were reuspended in A100 + 10% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA to a final total lipid concentration of ~10 mM. Large unilamellar vesicles were formed by 10 freeze–thaw cycles in liquid N₂ and room temperature water. Uniform-sized large unilamellar vesicles were formed by extrusion through polycarbonate filters with 100-nm pore size (Avanti Polar Lipids).

**Protein Expression and Purification** – GST-tagged proteins were purified starting by transforming E. coli BL21(DE3) (Stratagene) cells with the respective expression plasmid. Overnight colonies were picked to start pre-precultures in 5 ml LB + Amp. The pre-precultures were used to start 50 ml pre-cultures that were grown overnight at 25°C. The cells from the 50 ml pre-cultures were pelleted at 3,000 rpm (~2,000xg) for 15 min using an Allegra tabletop centrifuge at room temperature. The cell pellet was used to seed 2 to 4 L cultures with a starting density of about 0.1 to 0.2 optical density at 600 nm (OD). The cultures were grown at 25°C up to a desirable density (OD = 0.4 to 0.6) at which time the culture was moved to a 16°C incubator. Ten minutes after growth at 16°C, the protein expression was induced with 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and the cultures were grown overnight (~16 hours) at 16°C. The cells were harvested, washed with A200 (25 mM HEPES (pH 7.4), 200 mM KCl) and lysed in breaking buffer (A200 + 10% glycerol, 2 mM 2-mercaptoethanol (βME), 4% Triton X-100 and 1X complete protease inhibitor cocktail (Roche)) by passage through an Emulsiflex C-3 high pressure homogenizer (Avestin) twice at 15,000- 20,000 psi. Cell extracts were cleared by centrifugation at 125,000 x (~186,000xg) for 30 min using a Type 45-Ti rotor in an Optima LE-80K ultracentrifuge (Beckman) cooled to 4°C. Cleared extracts were incubated with Ni Fast Flow beads for 30 mins at 4°C. The Ni bead-bound protein was washed with 10mL of A400 (25 mM HEPES (pH 7.4), 400 mM KCl) + 10% glycerol, 2 mM βME and 1% Triton X-100. The cells were then washed with 10mL of A100 (25 mM HEPES (pH 7.4), 100 mM KCl) + 10% glycerol, 2 mM βME and 1% Triton X-100 and then washed again with 10 mL of A100 + 10% glycerol, 2 mM βME, 1% Triton X-100 and 0.1M Imidazole. The Ni bound protein was then eluted with A100 + 10% glycerol, 2 mM βME, 1% Triton X-100 and 0.1M Imidazole twice. The elutions were combined and incubated with 90 mg of swelled glutathione (GSH)-agarose beads (Sigma) for 1 hr at 25°C. The GSH bead-bound protein was washed with A100 + 10% glycerol, 2 mM βME, 1% Triton X-100, and 1 mM EDTA. The protein was washed 5 more times with 10 ml of A100 + 10% glycerol, 2mM βME, 0.1% anapoe X-100, and 1mM EDTA. The protein was eluted from GSH-agarose with A100 + 10% glycerol, 2 mM βME, 0.1% anapoe X-100, 1 mM EDTA, and 10 mM GSH.
Proteins were analyzed by SDS-PAGE and concentrations were determined by the amido black protein assay (16). Frozen aliquots were stored at -80°C.

Reconstitution— Atlastin and Atlastin mutants were reconstituted in both labeled and unlabeled 100-nm liposomes by a detergent-assisted insertion method (17,18) as follows. dAtlin 0.1% Anapoe X-100 was mixed with preformed liposomes at a 1:400 protein-to-lipid molar ratio and an effective detergent-to-lipid ratio of ~0.8. The effective detergent-to-lipid ratio (R_{eff}) was determined by the equation R_{eff} = D_{total} - D_{water}/[lipid] where D_{total} is the total detergent concentration and D_{water} is the monomeric detergent concentration (0.18 mM) in the presence of lipid. Protein and lipid were allowed to mix for 1 h at 4 °C. Detergent was removed by Bio-Beads SM-2 Adsorbent (Bio-Rad) at 70 mg of Triton X-100 per 1 g of beads. Insoluble protein aggregates were pelleted by centrifugation of the samples in an Eppendorf microcentrifuge [10 min at 16,000 × g (max)]. Final lipid and protein concentrations were determined by liquid scintillation counting and the amido black protein assay (16), respectively. Protein:lipid ratios ranged from approximately 1:400 to 1:600 and protein concentrations ranged from approximately 1.5 to 2.5 µM. Reconstitution efficiencies were not dependent on protein or lipid composition.

In vitro Fusion Assays— Fusion assays were based on a previously described method (6) with the following modifications. Labeled and unlabeled populations of Atlastin or mutant Atlastin proteoliposomes (0.15 mM lipid each) were mixed in the presence of 0.5 mM GTP or buffer only and brought to total volume of 45 µL with A100 buffer in wells of 96-well FluoroNunc PolySorp plates (Nunc). The total volume was adjusted to 50µl with A100 buffer. Blank samples contained liposomes, but no peptide. All the samples were excited at 278 nm in spectrofluorometer (Infinite M200; Tecan) and emission was measured between 300 to 500nm.

Cell Culture and atlastin Knockdown— HeLa cell culture, knockdown, and imaging were performed as previously described (19).

RESULTS

The Atlastin C-terminal Tail is an Amphipathic Helix and Interacts with Membranes— We have recently shown that the Atlastin C-terminal 23 amino acids are required for membrane fusion in vitro and in vivo (11). Bioinformatic analysis suggests that this segment of Atlastin is an amphipathic helix (11) and Figure 1A). We next wanted to probe this requirement more deeply by examining the interaction of a peptide corresponding to this region with membranes.

GTPase Measurements— GTPase activity was measured as previously described (6) by measuring the release of inorganic phosphate from GTP as suggested by the EnzChek Phosphate Assay Kit (Molecular Probes). Recombinant Atlastin and Atlastin mutants were mixed in a 100-µL reaction volume with 1 U/mL purine nucleoside phosphorylase (PNP), 200 µM 2-amino-6-mercaptop-7-methylpurine riboside (MESG), and GTP in a transparent 96-well plate. The plates were warmed to 37°C in a microplate reader (Infinite M200; Tecan), and 5 mM Mg^{2+} was added to start the reaction. In the presence of inorganic phosphate, PNP catalyzes the conversion of MESG to ribose 1-phosphate and 2-amino-6-mercaptop-7-methylpurine, resulting in a spectrophotometric shift in absorbance from 330 nm to 360 nm. The absorbance increase at 360 nm was measured in real time approximately every 20 s over 20 min. Absorbance was normalized to phosphate standards, and initial rates were calculated.

Measurement of Tryptophan Fluorescence— The dAtlin C-terminal tail peptide (45µM) and liposomes (9.5mM) were mixed in Greiner half area plate (black). The total volume was adjusted to 50µl with A100 buffer. Blank samples contained liposomes, but no peptide. All the samples were excited at 278 nm in spectrofluorometer (Infinite M200; Tecan) and emission was measured between 300 to 500nm.
The Atlastin amphipathic tail destabilizes membrane structure

phosphotidylcholine or a mixture of 85 mole% phosphotidylcholine and 15 mole% phosphotidylserine were incubated with or without peptide and intrinsic tryptophan fluorescence was determined. Figure 1B shows that the C-terminal peptide is membrane active and binds to both membrane compositions, with a slight preference for membranes with no charge (Figure 1B, green trace). These results are in agreement with a recent study that determined that a similar peptide from Drosophila Atlastin bound to membranes and could supply the required function in trans (20).

The Atlastin C-terminal Amphipathic Helix Perturbs Lipid Bilayers – Circular dichroism (CD, Figure 1D) suggests that the C23 is largely unstructured in solution (blue circles), even in the presence of liposomes (red circles). However, alpha helical secondary structure parallel to the surface of the bilayer is observed by oriented circular dichroism (OCD, Figure 1E) when the peptide is located between membranes of a multilayered structure.

We hypothesize that the primary function of the amphipathic C-terminal tail is to destabilize membrane structure during Atlastin-mediated membrane fusion. To test the effects of the C-terminal peptide on membrane structure, we employed X-ray diffraction to measure lipid organization in the presence of the peptide (21). Figure 1F-G shows characteristic changes to membrane structures that are consistent with bilayer perturbation. The presence of the peptide results in an overall bilayer thinning indicated by a reduced phosphate to phosphate distance with an increase in peptide concentration (Figure 1F and G), by about 10% (~38 to 34 Å). Additionally, the presence of the peptide resulted in a broadening of the trough of minimal X-ray intensity within the hydrophobic phase of the bilayer indicative of acyl chain disorder (Figure 1F). Overall, the Atlastin C-terminal tail peptide binds parallel to the surface of membranes as an alpha helix to induce bilayer thinning and acyl chain disorder, two properties important for the lipid rearrangements that occur during membrane fusion (1-3,22-24)

The Amphipathic Character of the C-terminal Juxtamembrane Tail of Atlastin is Conserved Across Species– We tested the functional conservation of the membrane perturbing properties of this sequence by constructing chimeric Drosophila Atlastins that contain the entire C-terminal tail of human Atlastin-1 or human Atlastin-3 (Figure 2). The loss of the tail eliminates fusion under most conditions (11,20), but the presence of the human tails restores in vitro fusion to approximately 50% of the wildtype protein. This level of recovery is comparable to replacing the entire 60 amino acids tail with the juxtamembrane conserved 23 amino acids (11). The ability of these Drosophila-human chimeras to support in vitro fusion confirms that the membrane destabilizing characteristics of the amphipathic helix is also conserved across species.

Alteration of the Hydrophobic Face of the Amphipathic Helix Reduces Membrane Fusion– The characteristic repeat structure of an amphipathic helix can be disrupted by replacing hydrophobic side chains that normally interact with the membrane with charged side chains. We generated several mutant Atlastins that alter the hydrophobic face, three of which are reported below. These include F478K, L482K and the double mutant (F478K; L482K). These mutants were produced, reconstituted into proteoliposomes, and fused. Introduction of a single charge (F478K) close to the membrane interface reduced homotypic fusion by about 35% (Figure 3A and C, green trace and histogram, Figure 6) while a single charge one turn further down the helix face (L482K) decreased fusion by ~80% (Figure 3A and C, red trace and histogram, Figure 6). The double mutant (F478K; L482K), was marginally more effective than either single mutant (~85% reduction, Figure 3A and C, blue trace and histogram, Figure 6). A mutation in the C-terminal tail, but not in the hydrophobic face, G480K, did not reduce the fusion activity of Atlastin (Figure 6).

Mutations in the hydrophobic face also impair the ability of the C-terminal tail to bind membranes. A peptide with the F478K and L482K mutations (C23-KK) shows reduced binding to liposomes (Figure 1C). The C23-KK peptide, like the wildtype C23 peptide, is unstructured in solution (Figure 1D). Unlike C23, C23-KK remains unstructured even when located between lipid bilayers. (Figure 1E).

Given that we conduct fusion reactions with equal amounts of protein and lipid in both the labeled and unlabeled liposome populations, we can also determine the effects of mutations on heterotypic membrane fusion. In these reactions,
The Atlastin amphipathic tail destabilizes membrane structure

The mutant Atlastin can be reconstituted in either the fluorescently labeled liposome population or the unlabeled bulk lipid liposome population. The location of the mutation relative to the fluorescent lipid labeled used to determine lipid mixing will influence the apparent effects on fusion. The schematic in Figure 3E illustrates these effects. When we measured heterotypic fusion with the L482K charge mutant, we found an intermediate level of fusion (Figure 3B, red traces with circles) compared with homotypic fusion (Figure 3B, red trace no symbols). The slight improvement in apparent fusion when the L482K mutation is located in the fluorescently labeled liposome population (Figure 3B, red trace, filled red circles) can be explained by the population nature of the fusion reaction (Figure 3E).

Next, we tested the effects of mutations or deletions of the Atlastin tail in vivo. Previous studies have examined Drosophila expression in mammalian cells (6,11,25), primarily for the inability of mutant Atlastins to produce an overexpression phenotype. However the functionality of Drosophila Atlastin in mammalian cells has not been demonstrated. HeLa cells primarily express human Atlastin-2 and Atlastin-3 with little or no Atlastin-1 (19). Knockdown of Atl-2 and Atl-3 by siRNA has proven to be a useful platform to examine Atlastin function by reintroduction of wildtype or mutant proteins (19). Loss of Atl-2 and Atl-3 in HeLa cells results in abnormal ER morphology manifested as elongated ER tubes with reduced three way junctions (19), Figure 4A). When wildtype, full length Drosophila Atlastin is reintroduced, normal ER morphology is restored (Figure 4B). The rescued ER morphology is due to Drosophila Atlastin function since it is responsive to a dominant GTP binding mutation (K51A), which does not remedy the aberrant ER morphology (Figure 4C). Drosophila Atlastin function in Hela cells also requires the C-terminal amphipathic tail. An Atlastin mutant truncated at residue 471 that removes the C-terminus, but maintains an ER retention signal, also fails to recover the ER morphology phenotype (Figure 4D).

Next we determined the effect of C-terminal tail mutations of the amphipathic alpha helix in vivo. Surprisingly, single charge mutations in the C-terminal tail were largely without effect in vivo (Figure 5A and B and Figure 6). We expanded the mutant collection to include multiple mutations down the length of the hydrophobic face. Loss of function required four charge replacements before in vivo recovery was lost (Figure 5D and Figure 6). Figure 6 compares the functional effects of point mutations within the hydrophobic face of the amphipathic helix both in vitro and in vivo.

**Lipid Composition Strongly Affects Requirements for the C-terminal Amphipathic Helix**– Taken together, these data confirm that the C-terminal tail is required in vivo, but its functionality is much less susceptible to small changes in the physical characteristics of the amphipathic helix. One possible explanation is that even a low level of fusion activity is sufficient to restore the ER network in vivo. An alternative, more likely, explanation is the substantial difference in lipid composition between the in vitro experiments and in vivo result. To test the latter hypothesis directly, we re-examined in vitro fusion with an altered lipid composition. We suggest that the fundamental role of the C-terminal helical region is to provide membrane destabilization during the fusion mechanism. This role is supported by effects of the peptide containing this region on structure. However, alterations in lipid composition can also influence the propensity of a bilayer to undergo the necessary lipid transitions during fusion. If the role of the C-terminal helix is to directly interact with the membrane to destabilize it, perhaps an inherently more unstable lipid mixture will alleviate the need for this activity.

We produced proteoliposomes that contained increasing concentrations of the non-bilayer forming lipid phosphatidylethanolamine (PE). The physical properties of PE and its relatively large amount of spontaneous negative curvature allow for membrane structural changes that are required during fusion (26). For this reason, membranes that contain PE are inherently more unstable and easier to fuse. Figure 7 shows the outcome of in vitro membrane fusion reactions with mixtures of wild type protein and the terminally truncated Atlastin that lacks the amphipathic tail. When wild type Atlastin is present in both liposome populations, the inclusion of PE at 10 mole% (green bar) or 20 mole% (blue bar) is largely without effect. However, as we have previously shown (11), when both liposome
The Atlastin amphipathic tail destabilizes membrane structure

populations contain the C terminally truncated Atlastin (1-471), fusion is largely abrogated in standard PC: PS liposomes (black bar). Fusion is marginally improved with 10 mole% PE to roughly 9% of wild type, while 20 mole% PE significantly improved in vitro fusion to roughly 31% of wild type. In this reaction, neither fusion partner contains the C-terminal tail yet fusion can occur at a reduced level. We also examined heterotypic fusion reactions that contained one C-terminal tail. The right half of Figure 7A shows these results. A heterotypic interaction with one C-terminal tail in the PC:PS mixture was improved, and fusion approached wild type levels when PE was included in the lipid mixture. As previously noted, the extent the fusion in the heterotypic reaction is dependent on the location of the mutant protein, in this case the C-terminal deletion, relative to the fluorescent lipid (Figure 3E). Reconstitution efficiency is not affected by the addition of PE (Figure 7B). The fusion activity of the L482K mutant was also improved by the addition of PE (Figure 7C).

DISCUSSION

Membrane fusion requires phospholipids and other membrane components to undergo transitions that are energetically unfavorable and contrary to normal bilayer structure. Resident membrane proteins are responsible for encouraging these transitions and ultimately driving membrane merger. Membrane fusion driven by the GTPase Atlastin requires energy in the form of GTP hydrolysis as well as a conserved C-terminal amphipathic tail. Here we examine the role of this surface active peptide during the process of membrane fusion. We found that a peptide corresponding to residues 470 and 492 of the Drosophila Atlastin interacts with membranes and does so in a manner consistent with bilayer perturbation (Figure 1). A recent study (20) found that a similar peptide (residues 479-507) also binds to membranes and can serve its required role when added in trans as a soluble peptide in an in vitro fusion reaction.

The amphipathic nature of the C-terminal tail is conserved across species and membrane perturbing activity is also transplantable from human Atlastin-1 and -3 to Drosophila Atlastin (Figure 2). A continuous hydrophobic face is required for normal Atlastin function since replacement of conserved hydrophobic side chains with charged residues reduces the ability of Atlastin to drive fusion in vitro (Figure 3); however, this requirement is less stringent in vivo (Figure 5). We found that Drosophila Atlastin can functionally replace human Atlastin-2 and -3 in Hela cells and restore ER morphology. However, a GTP binding mutant K51A, does not rescue (Figure 4C) nor does a truncated Atlastin lacking the conserved amphipathic tail (Figure 4D). The hydrophobic character of the amphipathic helix is also important in vivo, but much more significant perturbation is required to influence the ability of dAtl to rescue Atl-2/-3 knockdown (Figure 5 and 6). One likely reason for the decreased sensitivity to Atlastin tail mutation in vivo is the effect of membrane composition. Our in vitro studies utilized a very simple lipid composition, in most cases a single phospholipid. The endogenous lipid composition of the ER membrane (27) is much more complex and will influence membrane curvature and surface interactions among others. To test this possibility more directly, we altered the lipid composition of our in vitro fusion reaction to include increasing concentration of the non-bilayer forming lipid PE. The net effect of PE inclusion is to destabilize the membrane by providing an increased ability to undergo curvature transition. With increasingly destabilized bilayers, we found a decreased requirement for the C-terminal tail (Figure 7A). In essence, the destabilizing function of the C-terminal tail is unnecessary when the bilayer is composed of inherently unstable mixture.

The mechanical nature of the amphipathic C-terminal tail is also supported by the results of heterotypic fusion experiments. The graded fusion response with one functional C-terminal tail with increasing PE (Figure 7A, right) shows that bilayer perturbation is not required for both fusion partners. This is also true of the point mutations L482K in pure PC bilayers as well since a point mutant on either side of the fusion reaction yields an intermediate fusion response (Figure 3B). These observations show that mutations with the C-terminal tail are biochemically recessive, which is fundamentally different than mutations within the GTPase domain such as K51A or R48A which are biochemically dominant (6,11), consistent with the genetic dominance of homologous mutations in animals.
Membrane fusion driven by Atlastin appears to combine mechanistic features of both SNARE mediated fusion and type I virus-mediated fusion. Sequences within the transmembrane domain(s) are important (20) as are regions that perturb bilayer structure (Figure 1) such as viral fusion peptides (28). The GTPase requirement that likely drive conformational changes (29-31) necessary for fusion may be analogous to Rab proteins that influence the assembly of SNARE proteins (32). Future analysis of the atlastin fusion mechanism will most likely reveal new features of this unique process.
The Atlastin amphipathic tail destabilizes membrane structure

REFERENCES


The Atlastin amphipathic tail destabilizes membrane structure


The Atlastin amphipathic tail destabilizes membrane structure

FOOTNOTES
JAM is supported by the National Institutes of Health (GM101377), The Virginia and L.E. Simmons Family Foundation, and The Hamill Foundation. HWH is supported by the National Institutes of Health (GM55203) and the Robert A Welch Foundation (C-0991). TL is supported by the National Institutes of Health (GM107285).

FIGURE LEGENDS

FIGURE 1. The Atlastin C-terminal tail is an amphipathic helix that perturbs bilayer structure. A. the Atlastin C-terminus forms an amphipathic helix. A structural cartoon of Atlastin illustrating the C-terminal amphipathic alpha helix shown in a cyan ribbon diagram with the membrane facing hydrophobic side chains shown as yellow sticks. The amino acid sequence of the C23 peptide, composed of residues 470-492, is shown above the cartoon helix. The amino acid sequence of the C23-KK peptide, containing the F478K and L482K mutations, is shown above the C23 sequence. B. The C23 peptide binds to membranes. Intrinsic tryptophan fluorescence is shown at various wavelengths in the presence or absence of liposomes. The C23 peptide alone (blue trace) indicates the intrinsic tryptophan fluorescence of the peptide. A significantly increased tryptophan fluorescence is seen in the presence of liposomes (pure phosphatidylcholine liposomes, green trace, PC: PS liposomes, red trace). C. The C23-KK shows reduced binding to liposomes. Tryptophan fluorescence in the presence of liposomes is shown as a % of the no lipid signal. D. CD spectra of C23 with and without PCPS liposomes and C23-KK are shown in red, blue, and green dots respectively. Spectra imply C23 and C23-KK were unstructured in solution, or in the presence of liposomes. E. Raw OCD spectra of C23 or C23-KK mixed into the multilamellar PCPS bilayers in P/L=1/40. The OCD curve indicates that C23 formed an alpha-helical structure parallel to the bilayer surface. C23-KK remained unstructured even in presence of bilayers. F. peptide interaction perturbs bilayer structure. Electron density profiles of the bilayers determined by x-ray diffraction at various peptide to lipid ratios (P/L) is shown. Z is the distance from the bilayer center. G. peptide interaction thins the membrane. The phosphate peak to phosphate peak distance (PtP) is measured for a series of P/L. Each point is the average PtP of two independently prepared samples.

FIGURE 2. The Amphipathic character of the C-terminal tail is conserved across species. A. alignment of the C-terminal cytoplasmic tail of human Atlastins and Drosophila Atlastin. Highlighted portions show highly conserved residues following the transmembrane domain. B. kinetic fusion graph of unlabeled dAtl acceptor proteoliposomes fused with equimolar amounts of fluorescently labeled dAtl donor proteoliposomes. NBD fluorescence was measured at 1-min intervals, and detergent was added at 60 min to determine maximum fluorescence. WT GST-dAtl-His8 (black); GST-dAtl(AAtl1-tail)-His8 (green); GST-dAtl(Atl-3-tail)-His8, (red); GST-dAtl(1-471)-His8 (gray, solid); control reaction in the absence of GTP, (gray, dashed). In all cases, the same dAtl mutant is reconstituted into both liposome populations. All fusion experiments are n=6. C. the extent of fusion at 60 min is represented in a histogram as the percentage of fusion. Error bars: standard error of the mean (SEM).

FIGURE 3. Decreasing the hydrophobicity of the amphipathic tail reduces membrane fusion in vitro. A. kinetic fusion graph of unlabeled dAtl acceptor proteoliposomes fused with equimolar amounts of fluorescently labeled dAtl donor proteoliposomes. NBD fluorescence was measured at 1 min intervals, and detergent was added at 60 min to determine maximum fluorescence. WT GST-dAtl-His8 (black); GST-dAtl(F478K)-His8 (green) GST-dAtl(L482K)-His8 (red); GST-dAtl(F478K; L482K)-His8 (blue); control reaction in the absence of GTP, (gray). In all cases, the same dAtl mutant is reconstituted into both liposome populations. All fusion experiments are n=6. B. kinetic fusion graph of heterotypic fusion dAtl proteoliposomes fused with equimolar amounts of GST-dAtl(L482K)-His8 proteoliposomes. *represents labeled liposomes. dAtl*-dAtl, black; dAtl(L482K)*-dAtl(L482K), red; dAtl*-dAtl (L482K) (red, open circles); dAtl(L482K)*-dAtl (red, filled circles). C. the average extent of fusion at 60 min is represented for wild type and mutant atlastins. All experiments are n=6. D. GTPase activity is represented
as a histogram for all mutants. All experiments are n=3. E, a diagram of homotypic and heterotypic fusion. Unlabeled acceptor liposomes and shown in black. Labeled donor liposomes are shown in red. Lower apparent fusion is observed when mutant Atlastin in present only in unlabeled liposomes. Higher apparent fusion is observed when mutant Atlastin is present only in labeled liposomes. Error bars: SEM. Numbers in histograms = number of replicates.

FIGURE 4. Drosophila Atlastin can functionally replace human Atlastin in HeLa cells. Knockdown replacement assay. 48 h after transfection with a negative control construct (Myc-tagged DP1, A) or the indicated Venus-dAtl constructs, cells were transfected with siRNAs targeting ATL2 and ATL3. 72 h after knockdown, cells were fixed and stained using an antibody against the Myc (A) or Venus fluorescence (B-D) and viewed by confocal microscopy. A. HeLa cells transfected with siRNA targeting Atl-2 and Atl-3 show elongated ER tubules and reduced three way junctions examining transfected DP1-myc. B. Expression of wild Drosophila atlastin tagged N-terminally with Venus in these cells restores ER morphology. C. A GTP binding mutant (Venus-dAtl(K51A)) does not rescue the abnormal ER morphology of siRNA-treated cells. D. A mutant lacking the C-terminal amphipathic tail (Venus-dAtl(Δtail)), but maintaining an ER retention signal also fails to rescue the abnormal ER morphology. Scale bar: 10 µm.

FIGURE 5. Charge mutations in the C-terminal tail are more permissive in vivo. Knockdown replacement assay. 48 h after transfection with the indicated Venus-dAtl constructs, cells were transfected with siRNAs targeting ATL2 and ATL3. 72 h after knockdown, Venus fluorescence was viewed by confocal microscopy. A. Drosophila Atlastin with a charge mutation in the amphipathic C-terminal tail, Venus-dAtl(L482K), is functional in vivo and can rescue the abnormal ER phenotype in cells treated with siRNA targeting Atl-2 and Atl-3. B. dAtl with a different charge mutation in the C-terminal tail, Venus-dAtl(A486D), is also functional in vivo. C. dAtl with three charge mutations in the C-terminal tail, Venus-dAtl(L482K;A486D;L489D), is functional in vivo. D. dAtl with four charge mutations in the C-terminal tail, Venus-dAtl(L482K;A486D;L489D;F493D), does not rescue the ER morphology. Scale bar: 10 µm.

FIGURE 6. Summary of in vitro and in vivo activity of drosophila Atlastin C-terminal tail mutants. The left panel shows a cartoon of the C-terminal amphipathic helix with the amino acids forming the hydrophobic face highlighted in yellow. Seven mutations in the hydrophobic face of the C-terminal tail are shown below the cartoon. Amino acids highlighted in red add a positive charge and those highlighted in blue add a negative charge. The top five mutants have been tested in vitro while the bottom four have been tested in vivo. The right panel summarizes the in vitro fusion activity of these mutants and their ability to rescue ER morphology in HeLa cells. The efficiency of plasmid recovery was determined as previously described (19). Briefly, Atlastin knockdown cells were transfected with various recovery plasmids and the extent of branched ER determined by confocal microscopy. The fraction of cells that exhibited a branched ER morphology with Drosophila atlastin and atlastin mutants were determined and compared to recovery with human atlastin.

FIGURE 7. Lipid composition strongly affects requirements for the C-terminal amphipathic helix. The extent of fusion at 60 min is represented in a histogram as the percentage of wildtype. A. The left half of the graph shows homotypic fusion reactions where the same protein is present in donor and acceptor liposomes. The right half shows heterotypic fusion reactions where mutant dAtl is present in one pool of liposomes and wild type dAtl is present in the other pool. PC:PS (85:15 mole %), black; PC:PS:PE (75:15:10 mole%), green; PC:PS:PE (65:15:20 mole%), blue. Error bars are standard error of the mean (SEM) with the number of replicated (n) indicated in the histogram. Absolute levels of fusion (represented as a percentage of maximum fluorescence) ranged from 8.2-8.9% following background subtraction of ~0.9% found in the absence of GTP. The level of background fusion in the absence of GTP did not significantly differ with increased levels of PE. B. A Coomassie-stained SDS-PAGE gel of
The Atlasin amphipathic tail destabilizes membrane structure

proteoliposomes. Protein:lipid ratios and protein concentrations and largely unaffected by adding PE to the lipid mixes. Protein:lipid ratios ranged from 1:453 to 1:583 (protein concentrations of 2.2 µM-1.63 µM) in PCPS, 1:426 to 1:566 (2.34 -1.77 µM) in PCPS10%PE and 1:392 to 1:600 (2.54-1.42 µM) in PCPS20%PE. Similar reconstitution efficiencies were seen with the L-471 mutant in various lipid mixes. C. The fusion activity of the L482K mutant is similarly improved by increasing PE.
Figure 1

A. Amino acid sequence of C23-KK peptide

B. Tryptophan Fluorescence (Arbitrary Units) vs. Wavelength (nm)
- PC liposomes
- PCPS liposomes
- C23 peptide alone
- PC lipos + C23
- PCPS lipos + C23

C. Maximum Tryptophan Fluorescence (% of no lipid) for C23 and C23 KK
- C23
- C23 KK

D. Circular Dichroism (millidegrees) vs. Wavelength (nm)
- C23-KK
- C23
- PCPS lipos + C23

E. Oriented Circular Dichroism (millidegrees) vs. Wavelength (nm)
- C23 in PCPC multilayers
- C23-KK in PCPS multilayers

F. Relative Intensity vs. Phosphate to Phosphate distance (Å)
- Various protein to lipid ratios

G. Phosphate to Phosphate distance (Å) vs. Protein / Lipid
- Various protein to lipid ratios
Figure 2

A. Sequence comparison of Atl-1, Atl-3, and dAtl proteins.

B. Graph showing fusion percentage (% of maximum) over time (min) for dAtl, dAtl(Atl-1 tail), dAtl(Atl-3 tail), and dAtl no GTP. Data points are presented for n=19, n=7, and n=7.

C. Bar graph with fusion percentage (% of maximum) for dAtl, dAtl(Atl-1 tail), and dAtl(Atl-3 tail).
Figure 3

A

Fusion (% of Maximum)

Time (min)

B

Fusion (% of Maximum)

Time (min)

C

Fusion

Time (min)

D

GTPase (µM Pi / min / µM protein)

Time (min)

E

Heterotypic fusion

Mutant protein in the unlabelled acceptor population

Higher probability of donor-donor fusion

Less FRET relief

Homotypic fusion

Equal probability of acceptor-acceptor or donor-donor fusion

Uniform probe dilution (FRET relief)

Heterotypic fusion

Mutant protein in the labelled donor population

Higher probability of acceptor-acceptor fusion

More FRET relief

Higher apparent fusion

n=6 n=3

n=3
Figure 4
Figure 5

A

dAtl(L482K)

B

dAtl(A486D)

C

dAtl(L482K;A486D;L489D)

D

dAtl(L482K;A486D;L489D;F493D)
### Figure 6

<table>
<thead>
<tr>
<th>in vitro fusion</th>
<th>in vivo rescue</th>
<th>% of Wt</th>
<th>% of Atl efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAtl</td>
<td>100</td>
<td>98.1</td>
<td></td>
</tr>
<tr>
<td>K51A</td>
<td>5</td>
<td>~0</td>
<td></td>
</tr>
<tr>
<td>Δtail (1-471)</td>
<td>5 (&lt;5*)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>F478K</td>
<td>66</td>
<td>nd‡</td>
<td></td>
</tr>
<tr>
<td>F478K;L482K</td>
<td>16</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>L482K</td>
<td>21</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>A486D</td>
<td>&lt;5*</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>L489D</td>
<td>&lt;5*</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>L482K;A486D;L489D</td>
<td>nd</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>L482K;A486D;L489D;F493D</td>
<td>~0</td>
<td>~0</td>
<td></td>
</tr>
<tr>
<td>G480K</td>
<td>104</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

* Liu et al PNAS
‡ not determined