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Analysis of Arf1 GTPase-dependent membrane binding and remodeling using the exomer secretory vesicle cargo adaptor

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Summary

Protein-protein and protein-membrane interactions play a critical role in shaping biological membranes through direct physical contact with the membrane surface. This is particularly evident in many steps of membrane trafficking, in which proteins deform the membrane and induce fission to form transport carriers. The small GTPase Arf1 and related proteins have the ability to remodel membranes by insertion of an amphipathic helix into the membrane. Arf1 and the exomer cargo adaptor coordinate cargo sorting into subset of secretory vesicle carriers in the model organism *Saccharomyces cerevisiae*. Here, we detail the assays we used to explore the cooperative action of Arf1 and exomer to bind and remodel membranes. We expect these methods are broadly applicable to other small GTPase/effector systems where investigation of membrane binding and remodeling is of interest.

Keywords

GTPase; Arf1; membrane remodeling; membrane binding; membrane trafficking; membrane fission; membrane scission; cargo adaptor; coat protein

1. Introduction

Protein-membrane and membrane-dependent protein-protein interactions are important in membrane trafficking and cell signaling pathways. Cells employ many proteins, including small GTPases, cargo adaptors, and vesicle coats, that bind membranes to mediate the trafficking of cargo proteins by sculpting organelle membranes to generate transport vesicles and tubules. One prominent example is the small GTPase Arf1, a highly conserved regulator of membrane trafficking at the Golgi complex [1–3]. Upon activation at the membrane surface, Arf1 recruits many different effectors, including cargo adaptors and lipid modifying enzymes, to Golgi membranes where they carry out their function. Thus, Arf1 and its paralogs act as molecular switches to drive multiple Golgi membrane trafficking pathways.

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¹⁶Crucial negative controls for this assay include reactions with liposomes only, liposomes with Arf1-GDP, and liposomes with Arf1-GDP and exomer.

Some Arf1 effectors exhibit affinity for the membrane independent of binding to Arf1 [3–6]. One such effector is the exomer complex, which is responsible for sorting a subset of cargos at the *trans*-Golgi network (TGN) in the model organism *Saccharomyces cerevisiae* [7,8,4,9,10]. To effectively assay for membrane binding and Arf1-dependent membrane recruitment, we employed a liposome pelleting assay where proteins and liposomes were incubated and subjected to centrifugation [6]. Protein bound to liposomes becomes enriched in the pellet fraction. The relative amount of the protein in the supernatant and pellet fractions was determined by quantification of band intensity after SDS-PAGE analysis. Thus, the relative efficiency of membrane binding and recruitment of different protein constructs and mutants can be measured quantitatively.

In addition to recruiting cargo adaptors necessary for trafficking, Arf1 and other related small GTPases, such as Sar1, have been implicated in membrane remodeling, defined as inducing membrane curvature or otherwise deforming the membrane [11–16]. Upon activation through GTP-binding, Arf1 inserts its myristoylated N-terminal amphipathic helix into the cytoplasmic leaflet of the membrane [17–19], and this membrane insertion is what drives membrane remodeling. Several other factors are known to play a role in membrane remodeling, including protein crowding, protein scaffolding, and protein shape [20,21].

We were interested in determining what role, if any, Arf effectors such as the exomer complex might have in regulating or enhancing membrane curvature. Exomer is the only cargo adaptor known to sort proteins from the TGN to the apical plasma membrane, yet it only sorts ~1–5% of *S. cerevisiae* plasma membrane proteins and is not found in metazoans. Thus, we view Exomer as an important model system that should provide insight into how the bulk of proteins are sorted to the apical plasma membrane. Exomer is a heterotetramer consisting of two copies of Chs5 and any two members of four paralogous proteins known as the ChAPs (Chs5 and Arf1 binding proteins: Chs6, Bud7, Bch1, and Bch2). The ChAPs determine cargo specificity by binding directly to the cytoplasmic tails of cargo proteins, but appear to be structurally interchangeable within the complex [22,8,6,10,7,4].

To investigate whether exomer can cooperate with Arf1 to remodel membranes, we employed a method similar to one previously reported by the McMahon lab [15], in which different sized liposomes are separated by differential sedimentation. Thus, the products of a vesiculation reaction (small liposomes) remain in the supernatant fraction, while the substrates (large liposomes) are found in the pellet fraction. We modified the established protocol by including a fluorescent tracer lipid in our liposome preparations, facilitating quantification of the liposomes.

Here we describe the methodology necessary for the preparation of liposomes and performing the Arf1/effector membrane binding and remodeling assays.

2. Materials

2.1 Preparation of synthetic liposomes

1. Rotary evaporator (see Note 1).
2. 25 mL glass pear-shaped flasks.

3. Liposome extruder and 400 nm pore filters (Avanti Polar Lipids).
4. Glass syringes, sizes from 10 μ l to 500 μ l.
5. Chloroform, ACS grade.
6. Methanol, ACS grade.
7. Lipid stocks (see recipes in Table 1).
 - a. Folch Fraction I.
 - b. Synthetic and natural lipids (Avanti Polar Lipids – see Table 1).
 - c. DiR lipid (Life Technologies) or other fluorescent lipid tracer (see Note 2).
8. HK buffer: 20 mM HEPES pH 7.4, 150 mM potassium acetate.

2.2 Liposome pelleting / membrane binding assay

1. Purified myristoylated Arf1 (for protocol, see reference [23]) and purified Exomer protein constructs (see references [24,6] for exomer purification method), or other protein to be used in the assay.
2. “TGN” liposomes (described in this protocol), or liposomes of another relevant composition.
3. HKM buffer: 20 mM HEPES pH 7.4, 150 mM potassium acetate, 1 mM magnesium chloride.
4. Beckman Coulter Optima TLX ultracentrifuge with a TLA 100.3 rotor
5. 1.5 ml polyallomer tubes
6. SDS sample (gel-loading) buffer

2.3 Liposome vesiculation assay

1. Purified myristoylated Arf1 (described in 2.2.1), or other protein to be used in the assay.
2. Folch liposomes (described in this protocol).
3. HKM buffer: 20 mM HEPES pH 7.4, 150 mM potassium acetate, 1 mM magnesium chloride.
4. 0.5 M EDTA solution, pH 8.0
5. 40 mM $MgCl_2$

¹Alternatively, many researchers dry lipid mixes under a nitrogen stream.

²DiR, or a similar fluorescent lipid tracer, should be added at 1% (mol basis) for visualization and quantification of liposomes. The fluorophore should be chosen based on the available instrumentation. DiR works well with the Odyssey (Li-Cor) scanner. Non-fluorescent lipids can also be detected in the visible spectrum after staining with coomassie stain, but at lower sensitivity.

6. Guanine nucleotides (GDP and GTP, or the non-hydrolyzable GTP analogs GMP-PNP or GTP γ S), 10 mM solutions in water.
7. Beckman Coulter Optima TLX ultracentrifuge with a TLA 100.3 rotor
8. 1.5 ml polyallomer tubes
9. SDS sample (gel-loading) buffer

3. Methods

3.1 Preparation of synthetic liposomes

Different liposome compositions may be employed in liposome pelleting and vesiculation assays, and the liposome composition should be customized depending upon the protein(s) being investigated. To assay membrane binding of exomer constructs, we generally use a lipid composition intended to mimic the yeast TGN (Table 1). In contrast, for the vesiculation assay, it appears necessary to use Folch lipids. The Folch liposomes can also be used for the binding assay.

1. Make 0.5 – 4 ml of an appropriate lipid master mix, with a final concentration of 2 mM lipids dissolved in 20:1 chloroform:methanol (see Notes 3 and 4). See Table 1 for master mix formulations.
2. Transfer 500 μ l of the 2 mM lipid master mix to a 25 ml pear-shaped flask and dry on a rotary evaporator under vacuum, spinning the flask at an intermediate speed, for approximately 30 minutes at 30°C or until all of the solvent has evaporated from the sample, resulting in a thin lipid film on the bottom of the flask (see Notes 1 and 5).
3. Rehydrate the lipids by gently adding 1 mL of HK buffer to the lipid film and incubating at 37°C for 1–4 hours.
4. Gently swirl or rotate the flask so that the lipid film slowly peels off the glass and large strands or ribbons of lipids can be seen, continue until the lipids have been completely resuspended.
5. Using a mini-extruder (Avanti Polar Lipids), extrude the lipids through a sizing filter, extruding back-and-forth 19 times. We use 400 nm pore size filters for both the membranebinding (pelleting) and vesiculation experiments (see Note 6).

³Avoid using plastic tubes, pipets, and pipet tips with organic solvent solutions for this and subsequent steps. Glass vials and small glass syringes (Hamilton) should be used to avoid contamination of the solutions with dissolved plastic material.

⁴Lipid stocks in organic solvents should be stored tightly sealed at –20°C or ideally at –80°C.

⁵To store excess master mix, remove the organic solvent by evaporation and store the dried lipid film in tightly sealed glass vials at –80°C. To use at a later date, dissolve in the same volume of 20:1 chloroform:methanol solution.

⁶A 400 nm pore size is used to generate liposomes that are large enough to pellet and be used as substrates for vesiculation in the assays. It is important to note that extruding through pore sizes larger than 100 nm can result in liposomes that are multilamellar, rather than unilamellar. In the worst cases, this can lead to heterogeneity of lipid composition exposed on the outer surfaces of liposomes and inaccurate estimates of the amount of membrane surface available for protein binding. Drying the lipid mix in a pear-shaped flask on a rotary evaporator is intended to mitigate these effects by creating a very thin film prior to solubilization in aqueous buffer.

6. Transfer liposome solution to an eppendorf or conical tube and store at 4°C (see Note 7). The final concentration of lipids in the liposome solution is 1 mM.

3.2 Membrane binding (liposome pelleting) assay

This is a variation on the classical liposome pelleting assay, in which protein binding to membranes is monitored by measuring the fraction of the protein that pellets together with the liposomes. By measuring and subtracting the amount of protein that pellets in the absence of liposomes, we found that this assay can be quantified and yields results that are highly reproducible [24,6]. We present two variations of this assay, one to measure Arf1-independent membrane binding, and the other to measure Arf1-dependent membrane binding. By utilizing both versions of this assay, one can dissect the relative contributions of intrinsic membrane binding versus Arf1-binding for membrane recruitment of an Arf1-effector. An example result of this experiment is shown in Figure 1.

3.2.1 Arf1-independent membrane binding (liposome pelleting) assay—This protocol as detailed uses the Chs5/Chs6 exomer complex, but should be suitable for most membrane-binding proteins or protein complexes.

1. Mix liposomes (600 μ M final lipid concentration) with Chs5(1–299)/Chs6 exomer complex (1 μ M final concentration) in a final volume of 40 μ l HKM buffer in a 1.5 ml polyallomer tube (see Notes 8 and 9). Gently mix by flicking the tube.
2. Incubate at room temperature for 15 minutes. After 10 minutes gently mix by flicking the tube.
3. Spin the samples in Beckman TLX tabletop ultracentrifuge and TLA 100.3 rotor at 55,000 rpm (~150,000 g) for 10 minutes at 4°C. (see Note 10)
4. Remove the supernatant to another tube and add 10 μ l of 5 $\times$ SDS sample buffer. Add 50 μ l of 1 $\times$ SDS sample buffer to the pellet.
5. Heat the samples to 55°C for 15 minutes with frequent vortexing to mix, then run on SDS-PAGE. Do not run the dye-front off of the gel.

⁷Some researchers use liposome preparations only on the same day they were prepared, to avoid complications arising from lipid oxidation or phase changes. However, we find that our liposome preparations can be stored for a few weeks at 4°C without ill effects. It is important to determine whether the behavior of your liposome preparation changes after storage, by comparing results obtained with freshly prepared liposomes to results obtained using stored liposomes. Our practice is to prepare a large batch of liposomes at once (often 2–4 mL total) for use with large scale experiments performed over the course of 1 or 2 weeks. In our experience, this is preferable to preparing a fresh batch every day or even every few days, as we observe batch-to-batch variation in liposome behavior that is greater than variation in behavior observed after storage. We suspect this depends on the precise lipid mix and preparation method used, so it is best to test for your specific case.

⁸The concentrations given should be considered a starting point. You may need to alter the concentrations of proteins and lipid to optimize for your protein of interest.

⁹To account for any liposome independent (background) pelleting of your protein, a control in which no liposomes are included in the reaction should be used for each pelleting experiment.

¹⁰We have observed differences in the pelleting behavior of certain liposome compositions. 400 nm TGN and Folch liposomes completely pellet under these conditions, whereas 400 nm liposomes made up entirely of DOPC may not pellet as effectively. In such cases, longer centrifugation times may help. Also, if liposomes are densely coated with bound proteins, they pellet more effectively. This fact should be considered when interpreting the results.

6. Stain the gels using a coomassie dye solution lacking methanol or ethanol, or stain with LiCor IRDye colloidal coomassie solution.
7. Scan the gels on an Odyssey imager (Li-COR). Alternatively, another scanner instrument can be used (see Note 2).
8. Determine the protein band intensities using ImageJ or the Odyssey software. Note the presence of lipids from the liposomes in the dye-front. It is not necessary to quantify lipids in this experiment, but it is necessary to verify that virtually all of the lipids are in the pellet fraction.
9. Calculate the percent protein pelleted according to this formula:

$$\left[\frac{P_{\text{liposomes}}}{P_{\text{liposomes}} + S_{\text{liposomes}}} \right] - \left[\frac{P_{\text{no liposomes}}}{P_{\text{no liposomes}} + S_{\text{no liposomes}}} \right]$$
, where P= pellet, S= supernatant, and “no liposomes” represents the background level of protein pelleted in the absence of liposomes (see Note 11).

3.2.2 Arf1-dependent membrane binding (liposome pelleting) assay—A similar method can be used to determine the membrane-dependent protein-protein interaction between Arf1 and one of its effectors (such as exomer). For this protocol, Arf1 must first be activated (GTP-bound) in the presence of the liposomes to allow for membrane anchoring.

1. Mix liposomes (600 μM final lipid concentration) with Arf1 (7.5 μM final concentration), EDTA (1 mM final concentration), 125 μM GTP, GMP-PNP, or GTP γ S (to activate Arf1) or GDP (as a negative control) in a final volume of 40 μl HKM buffer in a 1.5 ml polyallomer tube. Gently mix by flicking the tube (see Note 12).
2. Allow the exchange reaction to continue for 15–30 minutes at room temperature.
3. Stop the exchange reaction by adding 2 μl of MgCl_2 stock solution to a final concentration of 2 mM.
4. Add exomer or other Arf1 effector to the reaction at a final concentration of 1 μM and mix by flicking the tube.
5. Follow steps 2 through 9 of the previous protocol (3.2.1). Do not run the dye front off of the gel. Ensure that virtually all of the lipids are present in the pellet fractions and not in the supernatant fractions. It is possible to observe lipid vesiculation with certain proteins under certain conditions. The appearance of lipids in the supernatant fraction is suggestive of vesiculation (see next section).

¹¹A key assumption to this analysis of the pelleting assay is that the same “background” amount of protein will pellet, presumably due to the presence of protein aggregates, in the presence or absence of liposomes. In our experience, this assumption is valid. For any new investigation of a protein by this method, it is important to pay close attention to the amount of background pelleting. Some proteins may exhibit a high level of background pelleting (i.e. >40% of the protein pellets in the absence of liposomes). This situation may be improved if the protein stock is centrifuged at high speed (150,000 g for 20 minutes at 4°C) to “pre-clear” aggregates (this is good general practice), prior to use in the membrane-binding reaction. For some proteins, this pre-clearing step does not prevent background pelleting and a liposome flotation experiment may be preferable. For a liposome flotation protocol, see reference [23].

¹²This step can be setup as several master mixes if different Arf1-effector constructs will be tested in parallel.

3.3 Liposome vesiculation assay

It has been previously observed that small GTPases of the Arf family can directly induce membrane deformation through insertion of the N-terminal amphipathic helix into the membrane [11–16]. To understand what role exomer might play in Arf1 membrane remodeling activity, we modified a previously described experiment from the McMahon lab [15] where the ability to observe changes in liposome size via protein dependent membrane remodeling was monitored using high speed centrifugation to separate liposomes of different sizes: larger liposomes (400 nm diameter) pellet, while smaller liposomes (below 100 nm diameter) remain in the supernatant. The lipids are visualized near the dye-front of the SDS-PAGE gel, taking advantage of a fluorescent lipid incorporated into the liposomes.

Overall, the vesiculation assay protocol is similar to the Arf1-dependent membrane pelleting assay described above in section 3.3.2, but there are important differences in the types of liposomes (see Note 13) and amounts of reagents used. We detail the protocol for the Chs5/Bch1 exomer complex, but other Arf1-dependent effectors can be investigated. The major strengths of this protocol are the ability to process many reactions in parallel and to quantitate the results. An example experimental result is shown in Figure 2.

1. For “physiological” conditions, where the ratio of exomer to lipids and exomer to Arf1 is low (1:1250 and 1:25, respectively, see Note 14) combine Folch liposomes (250 μ M final concentration, see Note 14) with Arf1 (5 μ M final concentration, see Note 15) and 125 μ M GTP, GMP-PNP, or GTP γ S (to activate Arf1) or GDP (as a negative control) in a final volume of 40 μ l HKM buffer in a 1.5 polyallomer tube. Gently mix by flicking the tube (see Note 16).
2. Allow the exchange reaction to continue for 10 minutes at room temperature.
3. Stop the exchange reaction by adding 2 μ l of $MgCl_2$ stock solution to a final concentration of 2 mM. Let the reaction incubate for an additional 5 minutes.
4. Add Chs5(1–299)/Bch1 exomer complex (200 nM final concentration) (See Note 14).
5. Let the reaction incubate 10 minutes at room temperature. Mix the reaction after 5 minutes and at the end of the incubation by flicking the tube.
6. Spin the samples at 20°C for 15 minutes in Beckman TLX tabletop ultracentrifuge and TLA 100.3 rotor at 55,000 rpm (~150,000 g).

¹³Vesiculation assays work best using liposomes prepared from Folch fraction I lipids. This lipid mix is currently the standard in the field for studying membrane remodeling, as Folch liposomes appear to be more amenable to remodeling.

¹⁴The concentration of the various constituents in a vesiculation assay can have a profound effect on the level of vesiculation. To further enhance the effects of Arf1-dependent membrane remodeling, we adjusted the exomer:lipids and exomer:Arf1 ratios. Exomer was used at 2 μ M final concentration and Folch liposomes were used at 50 μ M final concentration, increasing the ratios of exomer:lipids from 1:1250 to 1:25 and exomer:Arf1 from 1:25 to 1:2.5.

¹⁵This concentration of Arf1 results in a small but detectable amount of vesiculation in our hands, which is amplified by exomer. Higher concentrations of Arf1 result in higher levels of vesiculation.

7. Follow steps 4 through 7 from section 3.2.1 above.
8. Measure the lipid band intensities using ImageJ or ODYSSEY software to determine the percentage of total lipids found in the supernatant: $S/(S+P)*100$. The presence of lipids in the supernatant indicates a reduction in the size of liposomes, and thus vesiculation, assuming that no lipids are observed in the supernatant fractions of the negative controls (see Note 17).

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¹⁷Ideally, vesiculation should be confirmed using alternative approaches. For example, imaging the vesiculation reactions by negative stain-EM or analyzing the size distribution of liposomes by dynamic-light scattering [15,24].

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Figure 1. Detecting membrane-dependent protein-protein interactions using a quantitative liposome pelleting assay

Liposome pelleting assay to determine Arf1-dependent membrane recruitment of the exomer complex, Chs5(1–299)/Bch1. Mutations in Arf1 (N95Y and S98Y) at the Arf1-exomer interface were made to assess the ability of Arf1 to interact with exomer and recruit it to the membrane. Presence of protein in the pellet (P) fraction indicates an interaction with Arf1-GTP. S, supernatant. P, pellet. WT, wild-type. The left panel shows the gel, the right panel shows quantification. Band intensities were quantified using ImageJ. The fraction of Bch1 membrane bound was calculated using: $\frac{[P_{\text{liposomes (+Arf1)}} / (P_{\text{liposomes (+Arf1)}} + S_{\text{liposomes (+Arf1)}})] - [P_{\text{no liposomes}} / (P_{\text{no liposomes}} + S_{\text{no liposomes}})]}{[P_{\text{liposomes (+Arf1)}} / (P_{\text{liposomes (+Arf1)}} + S_{\text{liposomes (+Arf1)}})]}$, where the amount of Bch1 pelleted in the absence of liposomes is subtracted from the amount Bch1 pelleted in the presence of Arf1 and liposomes.

A

B

Figure 2. Vesiculation assay reveals that exomer and Arf1 cooperate to remodel membranes
 (A) Schematic of the membrane vesiculation assay. At high speeds, larger liposomes (~400 nm) will pellet completely, while smaller liposomes (~30 nm) will not pellet. A liposome population of mixed sizes can be spun at high speed and separated by size, with larger liposomes (and any bound proteins) in the pellet fraction and smaller liposomes remaining in the supernatant fraction. Lipids can be visualized via SDS-PAGE due to the presence of a fluorescent lipid dye in the liposomes. (B) Results of a membrane vesiculation assay to determine the role of exomer (Chs5(1–299)/Bch1) on Arf1-dependent membrane

remodeling. The left panel shows the gel and the right panel shows quantification of lipids. Substrate liposomes pellet (P), while smaller liposomes generated from protein-dependent membrane remodeling remain in the supernatant (S). $GTP^* = GMP-PNP$. Lipid band intensities were quantified using ImageJ and the percent of lipids found in the supernatant was calculated. Note that the high protein:lipid ratio results in a smaller fraction of total Arf1 binding to the membrane, and precise determination of the amount of Arf1 bound to membranes is confounded by vesiculation because Arf1 bound to smaller vesicles will remain in the supernatant.

Table 1

Lipid composition formulations “TGN” liposome lipid composition

Mol%	Lipid
24	DOPC
6	POPC
7	DOPE
3	POPE
1	DOPS
2	POPS
1	DOPA
2	POPA
29	PI
1	PI(4)P
2	CDP-DAG
4	PO-DAG
2	DO-DAG
5	Ceramide (C18)
10	Cholesterol
1	DIR

Folch liposome lipid composition

Mol%	Lipid
99	Folch Lipids (Folch Fraction I, Sigma)
1	DiR