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[13] Tethering Assays for COPI Vesicles Mediated by Golgins

By AYANO SATOH, JÖRG MALSAM, and GRAHAM WARREN

Abstract

A method is described that allows the attachment of COPI vesicles and Golgi membranes to glass slides that can then be analyzed using electron microscopy (EM) and immuno-EM methods. Subpopulations of COPI vesicles can be bound selectively using recombinant golgins. Alternatively, COPI vesicles can be attached to prebound Golgi membranes. Marking these vesicles selectively with biotin allows their site of attachment to be identified.

Introduction

The flow of material within the Golgi apparatus is mediated by COPI vesicles. Newly synthesized cargo proteins and lipids undergo serial modifications to the bound oligosaccharides as they move through this organelle. Large cargo is thought to move through cisternae that are remodeled around it, COPI vesicles removing one set of Golgi enzymes and bringing in the next set needed to carry out the modifications. Smaller cargo is thought to move in COPI vesicles, from one cisterna to the next, undergoing appropriate modifications in each. Simultaneous transport of different cargo therefore requires the coordinated action of different types of COPI vesicle, to ensure the correct intersection of cargo and enzymes in both time and space, so that each undergoes the correct sequence of post-translational modifications (Palade, 1975; Pelham and Rothman, 2000).

Determining the flow patterns for COPI vesicles has been hampered by the lack of available methods to subfractionate them biochemically, so that

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the origin and destination of each type can be determined using EM methods. One recent approach has been to exploit the specificity of membrane tethers to isolate subpopulations (Malsam *et al.*, 2005). These tethers are thought to help determine the destination of COPI vesicles, providing the initial attachment for the vesicle on the recipient cisternal membrane. There are many types of tethers, and we have focused on the golgin tethers, coiled-coil proteins that were originally identified as auto-antigens in patients with Sjögren's syndrome (Fritzler *et al.*, 1993). There are at least a dozen golgins, arrayed across the Golgi, so they could help provide the initial attachment at all levels of the stack (Barr and Short, 2003; Gillingham and Munro, 2003). Golgins interact with each other as well as directly (via transmembrane domains) or indirectly with membranes, as part of the tethering process (Shorter and Warren, 2002). Many also interact with small GTPases of the Ypt/Rab or Arl families (Barr and Short, 2003; Gillingham and Munro, 2003; Jackson, 2004).

A number of golgins have been characterized as well as several tethers, made up of different golgins. The latest is the CASP-golgin-84 tether that we recently showed is involved in the retrograde transport of Golgi enzymes within the Golgi stack (Malsam *et al.*, 2005). Both are membrane-anchored proteins (Bascom *et al.*, 1999; Gillingham *et al.*, 2002), golgin-84 in the COPI vesicle interacting with CASP on cisternal membranes (Malsam *et al.*, 2005). As part of the characterization process, we devised methods to reconstitute this tether attached to glass beads. This allowed us to study the composition of this subpopulation. We have also devised methods to attach COPI vesicles to glass slides coated with the cognate tether (in this case CASP) or Golgi membranes, so that the targeting site(s) of these vesicles could be ascertained. These are described in this chapter.

In Vitro COPI Vesicle Tethering Assay Using Purified Golgins

Materials

Purified rat liver Golgi: 300 μg (Hui *et al.*, 1997)
Rat liver cytosol (35% ammonium sulfate cut): 1 mg (Rabouille *et al.*, 1995)
Purified golgins: >1 mg/ml, ~10 μl (Satoh *et al.*, 2005)
Purified early endosomal antigen 1 (EEA1): >1 mg/ml, ~10 μl (Simonsen *et al.*, 1998)
Blocking reagents: BSA and soybean trypsin inhibitor (STI, Sigma, St. Louis, MO)
Assay buffer: 25 m*M* HEPES (pH 7.0), 2.5 m*M* magnesium acetate, 100 m*M* KCl, 1 m*M* DTT

GTP: 40 m*M* stock, neutralized with KOH
ATP-regenerating system (Rabouille *et al.*, 1995)
Glass slides: Esco Superfrost, precleaned (Erie Scientific Company, Portsmouth, NH)
Pap-pen (Zymed, San Francisco, CA)
All membranes and reagents should be prepared in, or buffer-exchanged to, assay buffer.

Methods

Preparation of COPI Vesicle-containing Supernatant

Rat liver Golgi membranes (300 μg) are incubated with rat liver cytosol (1 mg), 1 m*M* GTP, and an ATP-regenerating system in a final volume of 120 μl assay buffer for 10 min at 37°. The mixture is centrifuged at 14,000*g* at 4° for 10 min to remove larger membranes. The supernatants are used for the following tethering assay. They contain sufficient vesicles without the need to release more from the membranes using KCl (Malhotra *et al.*, 1989).

In Vitro *Tethering of COPI Vesicles to Golgin-Coated Glass Slides*

Microscope glass slides should be precleaned with 2 *M* NaOH for 30 min at room temperature, washed extensively with distilled water, and dried using an air duster. Circles should be drawn on the slides using a paraffin pen (Pap-pen, Zymed). Ten μl of recombinant proteins (1 mg/ml) are then allowed to attach for 30 min at 4° in a wet chamber. After washing with assay buffer, the slides are blocked with 1% BSA and 1% STI in assay buffer containing 9% (w/w) sucrose. Ten μl of the COPI vesicle-containing supernatant (above) is then placed in the circles and incubated in a wet chamber for 30 min at 4°. After washing, the samples are processed for EM (Seemann *et al.*, 2000). Briefly, the samples are embedded in Epon, which is then removed by immersion of the slide into liquid nitrogen for a couple of seconds followed by rapid warming using submersion into lukewarm water. Repeating this procedure several times will eventually remove all glass residues from the Epon surface. Blowing air onto the Epon surface using an air duster helps to get rid of all glass remnants and residual water. The Epon block is then cut parallel to the surface to generate 60 nm thick sections. For competition experiments, recombinant proteins (1 mg/ml final concentration) should be added prior to incubations on the coated slides. Results are quantitated as the number of vesicles/$\mu m^2 \pm$ S.D. Five areas on each of a total of three grids per condition should be counted.

An example is shown in Fig. 1. The input (Fig. 1F) contained a mixture of membranes including a minor fraction of uncoated COPI-sized

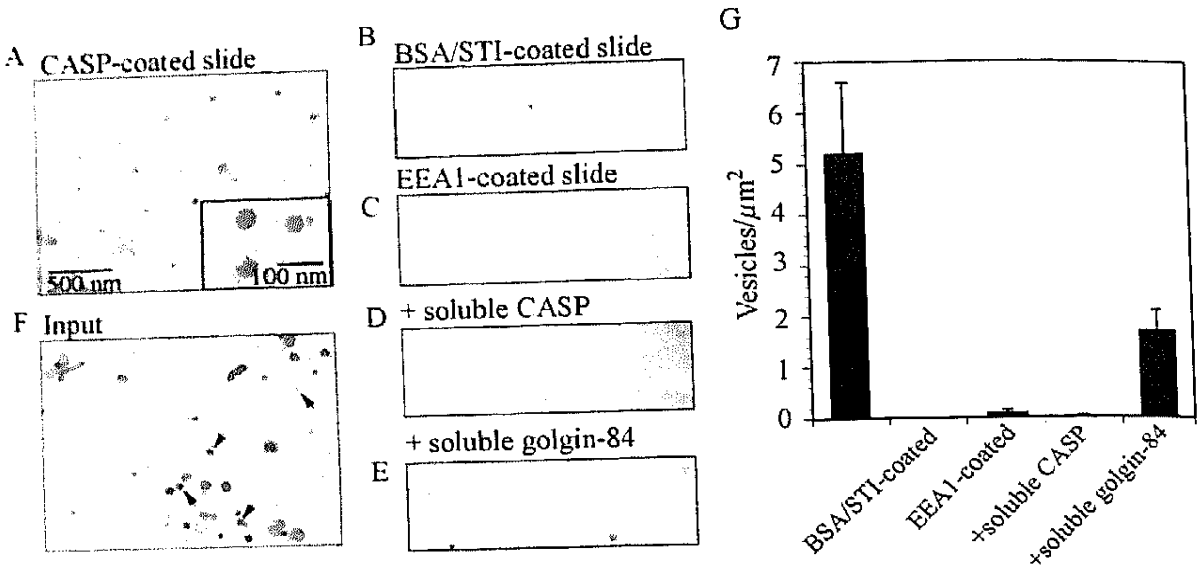

Fig. 1. Tethering of COPI vesicles to CASP-coated slides. (A–E) The vesicle-containing supernatant was incubated for 30 min at 4° on glass slides precoated with (A, D, E) recombinant CASP, (B) 1% BSA and STI, or (C) recombinant EEA1 in the absence (A, B, C) or presence of (D) soluble CASP or (E) soluble golgin-84. Glass slides were embedded in Epon resin and processed for conventional EM. (F) Input membranes. (G) Quantitation of the results in (A–E) presented as the mean of the number of the bound vesicles per $\mu m^2 \pm$ S. D.(n=5). (Reprinted with permission from *Science*.)

vesicles (~50 nm diameter, arrows). These were selectively bound to the CASP-coated slides (Fig. 1A) but not to those coated with BSA/STI or an irrelevant coiled-coil protein, EEA1 (Fig. 1B, C). The binding was specifically inhibited when carried out in the presence of soluble CASP or golgin-84 (Fig. 1D, E).

Note that uncoating the COPI vesicles is required for binding to golgin tethers (or Golgi membranes, see below) since binding was not observed when an Arf1Q71L mutant was used for preparation of COPI vesicles (Malsam *et al.*, 2005).

This method is not limited to CASP. Other tethers, such as GM130, have also been used to retrieve COPI vesicles from the vesicle-containing supernatant. GM130 binds to vesicles via p115 and giantin in the vesicle. In this case, p115-depleted cytosol should be used for the preparation of the COPI vesicle-containing supernatant since the cytoplasmic pool of p115 may mask the tether (giantin, in this case) on the vesicles (Malsam *et al.*, 2005).

Tethering of COPI Vesicles to Golgi Membranes Bound to Glass Slides

To approximate more the *in vivo* tethering of COPI vesicles to Golgi membranes, we have devised a method to attach Golgi membranes to glass slides and then incubate them with purified COPI vesicles. To distinguish the

added COP vesicles from the attached Golgi membranes they need to be marked. Serendipitously, this proved possible using a biotinylation reagent that, remarkably, labeled, almost exclusively, golgin-84 in COPI vesicles.

(A) Purification of Biotinylated COPI Vesicles

Materials

Purified rat liver Golgi: 1 mg (Hui *et al.*, 1997)
Sulfo-NHS-LC-biotin (sulfo-succinimidyl 6-(biotinamido) hexanoate; Pierce Chemical Co., Rockford, IL)
Purified rabbit liver coatomer: 200 μg (Pavel *et al.*, 1998)
Recombinant myristoylated Arf1: 200 μg (Franco *et al.*, 1995)
Assay buffer: 25 m*M* HEPES (pH 7.0), 2.5 m*M* magnesium acetate, 100 m*M* KCl, 1 m*M* DTT
GTP: 40 m*M* stock, neutralized with KOH
ATP-regenerating system (Rabouille *et al.*, 1995)
Beckman Vti 65.1 rotor, 6.3 ml tubes (#345830) and spacers (#349289)
All membranes and reagents should be prepared in, or buffer-exchanged to, assay buffer.

Methods

Biotinylation of Rat Liver Golgi Membranes

Golgi membranes (1 mg) are incubated with 0.5 μg/μl sulfo-NHS-LC-biotin for 1 h on ice in 200 μl assay buffer containing 500 m*M* KCl. The excess sulfo-NHS-LC-biotin is then quenched with NH_4Cl (final concentration 50 m*M*) for another hour on ice. The membranes are recovered by centrifugation at 13,000*g* at 4° for 10 min onto a sucrose cushion (10 μl of 23% (w/w) sucrose in assay buffer).

Purification of Labeled COPI Vesicles

The biotinylated Golgi membranes (~1 mg) are incubated with purified coatomer (200 μg), myristoylated Arf (200 μg), 1 m*M* GTP, and an ATP regenerating system in assay buffer containing 9% (w/w) sucrose in a final volume of 500 μl. After incubation for 10 min at 37°, 500 μl of 400 m*M* KCl in cold assay buffer is added to stop the reaction and release the vesicles generated. Large Golgi remnants are removed at 14,000*g* for 10 min at 4°. The supernatant is layered on a step gradient comprising 1.5 ml 30%, 1.5 ml 35%, 0.25 ml 37.5%, 0.25 ml 50%, and 2 ml 55% (w/w) sucrose in assay buffer containing 250 m*M* KCl. Membranes are centrifuged to equilibrium

at 400,000*g* at 4° for 2.5 h in a Vti65.1 vertical rotor. After puncturing the bottom of the tube, 0.5 ml fractions are collected as water is pumped (at 0.5 ml/min) on to the top of the gradient. COPI coated vesicles typically peak at 40–43% (w/w) sucrose (fractions 6–8) (Malhotra *et al.*, 1989). Coated cisternal remnants peak at about 35% (w/w) sucrose, and uncoated remnants peak at 30% (w/w) sucrose. The purity of the COPI vesicles can be confirmed by SDS-PAGE (an example is shown in Fig. 2A) and by electron microscopy by directly applying droplets of fractions on grids followed by

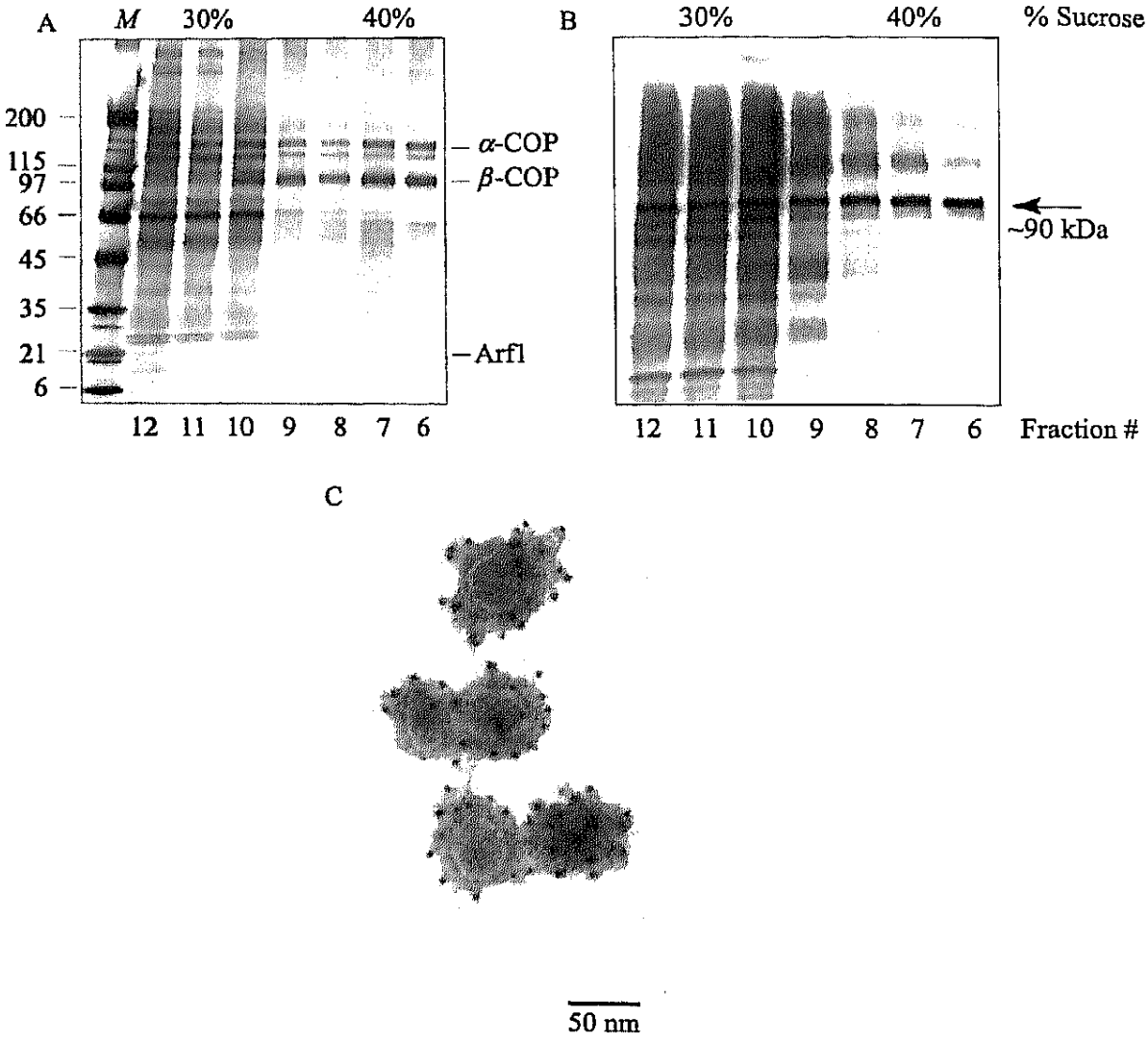

FIG. 2. Purification of biotinylated COPI vesicles. Biotinylated Golgi membranes were incubated with coatomer and Arf for 10 min at 37° to generate COPI vesicles. Membranes were centrifuged to equilibrium on a sucrose gradient and gradient fractions were analyzed by SDS-PAGE and (A) silver staining (M = Molecular weight markers) or (B) immunoblotting using anti-biotin antibodies. (C) Droplets from the gradient corresponding to 40% sucrose were processed for pre-embedding labeling using anti-biotin antibodies. Note that in the total population ~65% vesicles were labeled with anti-biotin.

negative staining, or immuno-labeled using antibodies to biotin followed by 10 nm Protein A-gold, and negatively-stained (an example is shown in Fig. 2C). The biotinylated protein on COPI vesicles (Fig. 2B, ~90 kDa) was identified as golgin-84 by MALDI-mass spectrometry.

Instead of biotinylation, fluorescently labeled COPI vesicles can be purified using this method. However, reagents such as NHS-Alexa-488 (Molecular Probes, A-10235) label many proteins in the COPI vesicles in addition to golgin-84.

(B) *In Vitro* Tethering of COPI Vesicles to Golgi Membranes

Materials

Purified rat liver Golgi: 1 mg/ml, ~10 μl (Hui *et al.*, 1997)
Rat liver cytosol (35% ammonium sulfate cut): 200 μg (Rabouille *et al.*, 1995)
Purified biotinylated COPI vesicles (above): ~500 μl
Blocking reagents: BSA and soybean trypsin inhibitor (STI, Sigma)
Assay buffer: 25 m*M* HEPES (pH 7.0), 2.5 m*M* magnesium acetate, 100 m*M* KCl, 1 m*M* DTT
GTP, ATP: 40 m*M* stock, neutralized with KOH
Glass slides: Esco Superfrost, precleaned (Erie Scientific Company)
Pap-pen (Zymed)
Beckman TLS55 rotor
All membranes and reagents should be prepared in, or buffer-exchanged to, assay buffer.

Methods

Microscope glass slides should be precleaned with 2 *M* NaOH for 30 min at room temperature, washed extensively with distilled water, and dried using an air duster. Incubation wells should be drawn on cleaned microscope glass slides using a Pap-pen. Ten μl of Golgi membranes are allowed to attach for 10 min at 4° and blocked with 1% BSA and 1% STI in 10 μl of assay buffer containing 9% (w/w) sucrose. Biotinylated COPI vesicles (~500 μl as collected from the gradient) are diluted with 2 volumes of assay buffer and transferred to a tube into which a small step gradient is introduced by sequentially underlaying 5 μl each of 38/40/45/50% (w/w) sucrose. After centrifugation at 100,000*g* in a TLS55 rotor for 1 h at 4°, around 10 μl of the visible band is sampled.

For uncoating, the concentrated COPI vesicles are incubated with cytosol (200 μg) in 120 μl assay buffer containing 1 m*M* GTP and 1 m*M* ATP for 10 min at 37°. BSA (1 mg/ml) is also added to this reaction to

prevent aggregation. Large membranes are then removed by brief centrifugation at 14,000*g*. After incubation with the vesicles in a wet chamber at 4° for 30 min, the slides are washed and fixed with 8% (w/w) buffered PFA, containing 10% (w/w) sucrose, and processed for immuno-EM (Satoh *et al.*, 2003).

Figure 3 shows examples of the types of results that have been obtained. Biotinylated COPI vesicles (5 nm gold) bind to cisternal membranes containing CASP (Fig. 3A, 10 nm gold) and p24 (Fig. 3B, C, 10 nm gold), the

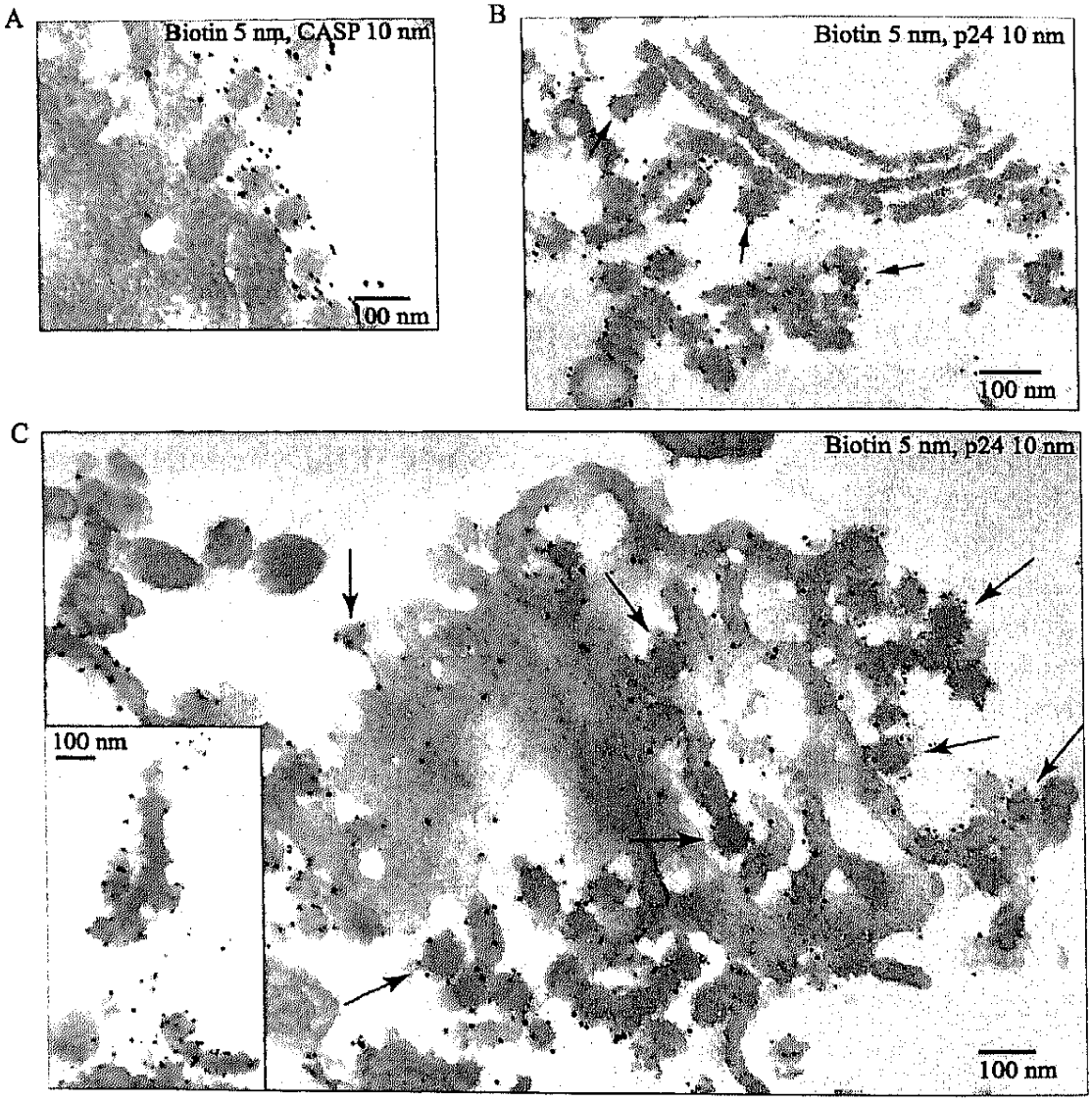

Fig. 3. Tethering of COPI vesicles to Golgi-coated slides. Biotinylated COPI vesicles were purified and incubated with cytosol for 10 min at 37° to allow uncoating. (A–C) Vesicles were incubated for 30 min at 4° on glass slides precoated with purified Golgi membranes (Inset in C). Additional incubation for 30 min at 25° was carried out to allow membrane fusion. After washing, slides were fixed and double-labeled for biotin (5 nm gold) and either CASP (A) or p24 (B, C, and inset) (both 10 nm gold), and processed for EM. Arrows in (B and C) indicate biotinylated COPI vesicles, binding to p24-containing membranes.

latter identifying them as cis-Golgi membranes (Rojo *et al.*, 1997). Warming the slides to 25° for 30 min led to fusion of the vesicles with the membrane (Fig. 3C, inset) showing that biotinylation had not inactivated them.

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[14] Investigating the Role of ADP-Ribosylation Factor 6 in Tumor Cell Invasion and Extracellular Signal-Regulated Kinase Activation

By Holly Hoover, Vandhana Muralidharan-Chari, Sarah Tague, and Crislyn D'Souza-Schorey

Abstract

Tumor cell invasion is a coordinated process involving the formation of invadopodia and the localized degradation of the extracellular matrix (ECM). The process of cell invasion is regulated by cell-signaling proteins such as Ras-related GTPases and members of the mitogen-activated protein kinase (MAPK) family. Our studies have focused on the role of the ADP-ribosylation factor 6 (ARF6) GTPase in the process of tumor cell invasion. Using activated and dominant negative mutants of ARF6 in a tumor cell culture model, our laboratory has demonstrated that the GTPase cycle of ARF6 regulates invadopodia formation and matrix degradation. Furthermore, ARF6-mediated cell invasion was found to be dependent on the activation of the extracellular signal-regulated kinase (ERK). These findings demonstrate a critical role for ARF6 in ERK activation and tumor cell invasion.

To investigate the role of ARF6 in tumor cell invasion and ERK activation, a number of methods were employed. These procedures include transfection of LOX cells, *in vitro* matrix-degradation assays, immunofluorescence microscopy, and biochemical assays. These approaches can be applied effectively to measure the degree of invasiveness fostered by

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