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Cytosol-derived proteins are sufficient for Arp2/3 recruitment and ARF/coatomer-dependent actin polymerization on Golgi membranes

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Abstract The actin cytoskeleton has been implicated in protein trafficking at the Golgi apparatus and in Golgi orientation and morphology. Actin dynamics at the Golgi are regulated in part by recruiting Cdc42 or Rac to the membrane through a binding interaction with the coatomer-coated (COPI)-vesicle coat protein, coatomer. This leads to actin polymerization through the effector, N-WASP and the Arp2/3 complex. Here, we have used reconstitution of vesicle budding to test whether Arp2/3 is recruited to membranes during the formation of COPI vesicles. Our results revealed that ARF1 activation leads to greatly increased Arp3 levels on the membranes. Coatomer-bound Cdc42 and pre-existing F-actin are important for Arp2/3 binding. ARF1-dependent Arp2/3 recruitment and actin polymerization can be reconstituted on liposomal membranes, indicating that no membrane proteins are necessary. These results show that activated ARF1 can stimulate Arp2/3 recruitment to Golgi membranes through coatomer, Cdc42 or Rac, and N-WASP.

Keywords: Golgi apparatus; Cytoskeleton; Cdc42; Coatomer; ADP-ribosylation factor; Arp2/3

1. Introduction

The actin cytoskeleton plays an important role in protein trafficking within the secretory pathway. Actin, numerous actin-binding proteins, and myosin motors are found to associate with Golgi membranes and Golgi-derived vesicles [1–6]. Functional studies indicate that actin-binding proteins and myosin motors function during protein transport between the Golgi and the ER [3,6,7]. Importantly, addition of actin toxins disrupts both anterograde and retrograde protein transport within or from the Golgi apparatus [8,9].

Actin dynamics at many sites in the cell, including the Golgi, involves the function of the Arp2/3 complex, a catalyst of actin polymerization. The Arp2/3 complex, constituting the two actin-related proteins (Arp2 and Arp3) and five other unrelated proteins (ARPC1-5), catalyzes actin polymerization [10,11]. Arp2/3 catalyzes actin polymerization by providing a nucleation site for growth in the barbed direction. It is intrinsically inactive and responds to upstream signaling pathways, i.e., through the GTP-binding protein Cdc42 and the WASP-family proteins, to allow spatial and temporal regulation of actin dynamics. The complex also has an important property of binding to pre-existing actin filaments causing the formation of branched actin structures.

Arp2/3 has been characterized mostly for its function in actin-based cell motility and comet-tail like movement of pathogens. Arp2/3 was recently found to be required for reorientation of the Golgi apparatus toward the leading edge when directed cell migration was induced by a scratch wound [12]. Arp2/3 as well as Cdc42 and WASP mediated signaling have been implicated in protein trafficking between the endoplasmic reticulum and the Golgi apparatus [3,13,14]. At the Golgi apparatus, Arp2/3-dependent actin polymerization can be regulated through a binding interaction between Cdc42 and the vesicle coat protein, coatomer [3,14]. The coatomer/Cdc42 complex is recruited to the Golgi upon activation of ARF1. Actin can affect the release of coatomer-coated (COPI) vesicles in vitro [2]. Cdc42 mediates the recruitment of the Arp2/3 activating protein, N-WASP, to the Golgi membrane [13]. These studies strongly implicate regulated Arp2/3 function in vesicle trafficking at the Golgi.

The role and regulation of Arp2/3-dependent actin polymerization at the Golgi complex remain to be fully characterized. In this study, we present evidence that Arp2/3 localization to the Golgi apparatus involves the functions of ARF1 and the coatomer-dependent recruitment of Cdc42 or Rac to the membrane. These findings closely link Arp2/3-dependent actin dynamics to the protein machinery that regulates protein trafficking.

2. Materials and methods

2.1. Materials

Rat-liver Golgi membranes and bovine-brain cytosol were isolated as described previously [15]. Actin in bovine-brain cytosol was depleted by binding to DNase I agarose [16]. *Clostridium difficile* toxin B (Tech Lab Inc., Blacksburg VA), p23 peptide (Sigma), latrunculin A (Molecular Probes), brefeldin A (Calbiochem), and GTPγS (Boehringer–Mannheim) were obtained commercially. Rabbit skeletal muscle actin was the generous gift of Kuo-Kuang Wen and Peter Rubenstein (University of Iowa). The glutathione *S*-transferase (GST)-fusion protein containing the CA domain of rat N-WASP was expressed and purified as described previously [3]. Recombinant myristoylated ARF1(Q71L) was expressed and purified using DEAE–sephacel

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[17,18]. The following antibodies were used in these studies: anti-actin (Sigma), anti-β-COP (Sigma), anti-Cdc42 (Upstate), anti-drebrin (MBL), sheep anti-Arp3 (Cytoskeleton), anti-ε-COP [19], anti-NWASP (Cytoskeleton), and anti-mAbp1 [3].

2.2. Golgi-binding reaction and membrane float-up assay

Golgi-binding reactions contained 25 mM HEPES (pH 7.2), 2.5 mM magnesium acetate, 15 mM potassium chloride, 0.2 M sucrose, Golgi membranes (0.2 mg/ml), bovine-brain cytosol (1.0 mg/ml), and an ATP regenerating system. The reactions were incubated for 20 min at 37 °C as previously described [2]. The final reaction volumes were 1 ml. When specified, GTPγS and/or the various inhibitors of actin signaling were added at the indicated concentrations. For reactions containing brefeldin A, the membranes and cytosol were pre-incubated for 10 min at 37 °C with the toxin or with a solvent control. Following the incubation, the membranes were isolated by centrifugation at 15 000 × *g* for 20 min at 4 °C in a refrigerated microcentrifuge. The membranes were then resuspended in 50 μl of 45% w/w sucrose in 25 mM HEPES, pH 7.2, and 25 mM potassium chloride and placed into a 7 × 20 mm ultracentrifugation tube. The sample was overlaid with 125 μl of 35% w/w sucrose in 25 mM HEPES, pH 7.2, 25 mM potassium chloride and then 25 μl of 15% w/w sucrose in 25 mM HEPES, pH 7.2, and 25 mM potassium chloride. The sample was spun at 100 000 rpm for 45 min in a TLA-100 rotor (Beckman Instruments). A 100 μl sample containing the purified Golgi membranes was removed from the top of the step gradient and the membrane-bound proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 10%. The trichloroacetic-acid precipitate was analyzed by SDS–PAGE and Western blotting.

2.3. Preparation of liposomes and liposome binding assay

Liposomes were prepared according to MacDonald et al. [20] with the following modifications. Rat liver lipid extract (Avanti Polar Lipids, Alabaster, AL) in chloroform was dried by evaporation and then hydrated at 2.32 mg/ml in 20 mM HEPES (pH 7.2), 100 mM potassium acetate, and 250 mM sucrose. The liposomes were suspended using a mechanical shaker and subjected to 10 freeze–thaw cycles. The vesicles were extruded through a polycarbonate membrane with a pore diameter of 400 nm (Avestin, Ottawa, Canada) to produce a unilammelar liposome population of uniform size [20]. For the liposome-binding assay, incubation conditions were identical to those used for the Golgi-binding reaction described above except that Golgi membranes were replaced with the liposomes (0.23 mg/ml). Following the incubation, the membranes were isolated by centrifugation at 15 000 × *g* for 30 min at 4 °C. The membranes were then washed two-times with 25 mM HEPES (pH 7.2) and 25 mM potassium chloride. The liposome-bound proteins were characterized by Coomassie-stained gels and mass spectrometry, or analyzed by Western blotting.

2.4. Mass spectrometry

Gel-resolved proteins were digested with trypsin and the mixtures fractionated on a Poros 50 R2 RP micro-tip [21]. Resulting peptide pools were then analyzed by matrix-assisted laser-desorption/ionization reflectron time-of-flight mass spectrometry using a Reflex III instrument from Bruker Daltonics (Bremen, Germany). Selected mass values were taken to search the protein non-redundant database (NR; National Center for Biotechnology Information, Bethesda, MD) using the PeptideSearch [22] algorithm.

3. Results

3.1. Arp2/3 is an ARF-dependent Golgi-binding protein

Previous studies have shown that the ARF-dependent assembly of COPI vesicles on Golgi membranes leads to concomitant actin polymerization [2,23]. This actin assembly involves the Arp2/3 complex and results in large part from the recruitment of Cdc42 through a binding interaction with coatomer. Actin dynamics at the Golgi could rely on activation of pre-bound protein machinery on the membranes or alternatively through the regulated association and disassociation of Arp2/3 and other cytoskeletal signaling proteins to the Golgi apparatus. In this regard, we examined whether the Arp2/3 complex binds Golgi membranes in an ARF-dependent manner.

We used a float-up Golgi-binding assay for these studies that alleviates artifacts that result from non-Golgi-bound cytoskeletal elements which co-sediment with the membranes in assays that rely on sedimentation [2]. Our results confirm that activation of GTP-binding proteins with GTPγS causes Cdc42 and Rac to be recruited to the Golgi membranes in addition to the COPI coat protein coatomer (Fig. 1A and B). This results in the polymerization of actin on the membranes (Fig. 1A). In vitro, Cdc42 and Rac often behave similarly and both are recruited to Golgi membranes upon ARF1 activation while, a third family member, RhoA, is not [3]. Cdc42 localizes to Golgi membranes in whole cells and is thus likely to be involved in Golgi function [24,25]. We used an antibody against Arp3 to determine the levels of the Arp2/3 complex on the Golgi membranes. In the absence of GTPγS, no Arp3 was bound to the Golgi membranes following incubation with cytosol (Fig. 1). When Golgi membranes were incubated with cytosol and GTPγS, Arp3 was clearly detected on the membranes (Fig. 1A and B). The membrane-bound Arp3 was derived from the cytosol, since Arp3 levels were below detection limits on the input membranes (data not shown). Blocking ARF activation with brefeldin A inhibits the GTPγS-dependent binding of Arp3 to the Golgi (Fig. 1A, lane 3), indicating that ARF is necessary for Arp2/3 recruitment to Golgi membranes. These results suggest that Arp2/3 binding is stimulated upon the ARF1-dependent recruitment of coatomer-bound Cdc42 (or Rac) to the Golgi membrane.

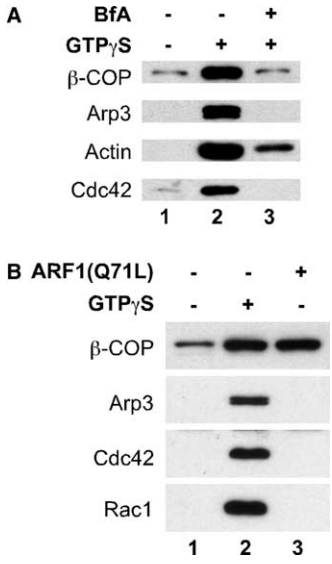

Fig. 1. (A) ARF is necessary but not sufficient for Arp2/3 recruitment to Golgi membranes. Shown is a Western blot of float-up Golgi-binding assays. GTPγS (20 μM) and 200 μM brefeldin A, BfA, were added to the incubations as indicated. The blot was probed with antibodies against β-COP, Arp3, actin and Cdc42. (B) Shown is a Western blot of float-up Golgi-binding assays. GTPγS or 40 μg/ml recombinant ARF1(Q71L) was added to the incubations as indicated. The blot was probed with antibodies against β-COP, Arp3, actin, Cdc42, and Rac1.

3.2. Arp2/3 recruitment involves signaling through coatomer/Cdc42

The constitutively active mutant ARF1(Q71L) recruits coatomer to the Golgi membranes but unlike GTPγS, does not induce Arp3, Cdc42 or Rac recruitment (Fig. 1B). This demonstrates that active ARF is not sufficient for Arp2/3 recruitment and that Cdc42 (or Rac) must also be activated, i.e., with GTPγS. We further tested whether Arp2/3 binding to the Golgi requires coatomer-associated Rho GTPases by using a peptide corresponding to the coatomer-binding domain of p23/p24 cargo proteins known to disrupt the interaction between γ-COP and Cdc42 [3,14]. We observed that Arp3 levels on the Golgi membranes were greatly decreased by the addition of p23 peptide to the reaction (Fig. 2B). By contrast, the levels of coatomer were unaffected by the peptide. Addition of the Rho-family GTPase inhibitor, *C. difficile* toxin B, provided additional evidence that Arp2/3 binding to Golgi is mediated through Cdc42 or Rac. Toxin B inhibited GTPγS/ARF1-dependent Arp2/3 binding to the Golgi membranes but had no effect on coatomer (Fig. 2C). These results indicate that the coatomer/Cdc42 interaction is necessary for the Arp2/3 recruitment to the Golgi membranes.

Cdc42 most likely leads to Arp2/3 activation on the Golgi apparatus by binding and activating N-WASP [3,13]. In this regard, we examined N-WASP binding to the membranes. Fig. 2D shows that N-WASP binds to Golgi in a GTPγS-dependent, Toxin B-sensitive manner. We used a GST fusion protein carrying the inhibitory C-terminal Arp2/3-binding domain (CA) of N-WASP to test if activation of ARP2/3 is necessary for Golgi binding [26–28]. Titration of the N-WASP-CA domain into the binding reaction caused dose-dependent inhibition of the GTPγS/ARF1-dependent Arp2/3 binding to the Golgi membranes, but had little effect on coatomer binding (Fig. 2E). GST alone affected neither coatomer nor Arp2/3 binding to Golgi (data not shown). These results indicate that activation of the Arp2/3 complex by WASP family members is necessary for its binding to the Golgi membranes.

3.3. Arp2/3 binding requires actin

Previous studies indicate that full activation of the Arp2/3 complex by WASP/Scar proteins involves the formation of an Arp2/3 complex with G-actin and a pre-existing "mother filament" [29–31]. This, together with our observation that only activated Arp2/3 bound to the Golgi membranes (Fig. 2E), led us to determine whether actin is necessary for recruitment of the Arp2/3 complex. We tested this by adding an actin-monomer-binding toxin, latrunculin A, to the binding assay. Fig. 3A shows that the Arp2/3 binding was inhibited by latrunculin A in a dose-dependent manner. The binding was completely blocked by latrunculin A at concentrations above 1 μM. Similar results for Arp2/3 binding and actin assembly on

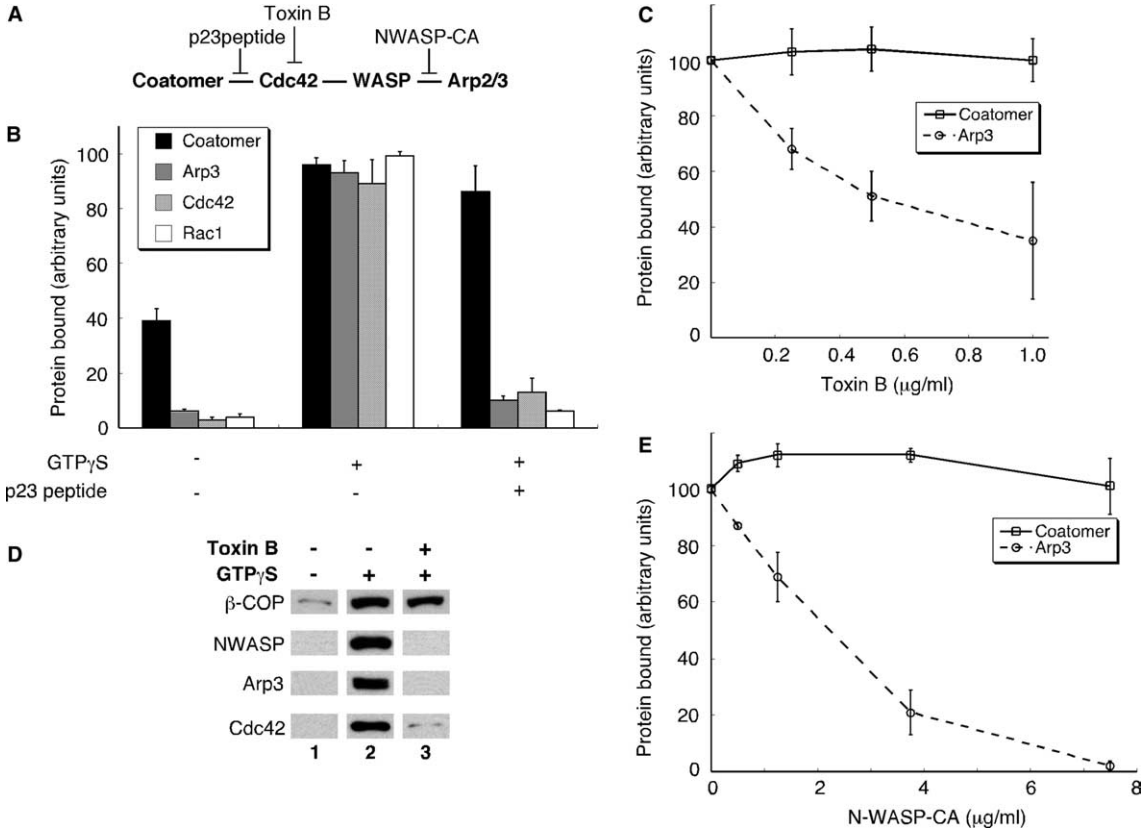

Fig. 2. Arp2/3 binding to Golgi membranes requires its activation via signaling through the coatomer/Cdc42 complex. (A) Shown is a diagram indicating the sites of action for the inhibitors of coatomer/Cdc42-mediated activation of WASP and Arp2/3 used in this study: N-WASP-CA, toxin B and the p23 peptide. (B–E) Shown are the results from float-up Golgi-binding assays carried out with 20 μM GTPγS, 250 μM p23 peptide (B), Toxin B (C and D), or recombinant GST-N-WASP-CA (E). Western blots of the isolated Golgi membranes were probed with antibodies against β-COP, Arp3, Cdc42, N-WASP, and Rac1 as indicated. In panels (B), (C), and (E), Western blots were quantified by densitometry and the average values from 3 (C) or 4 (B and E) experiments were plotted. The bars represent the standard error of the mean.

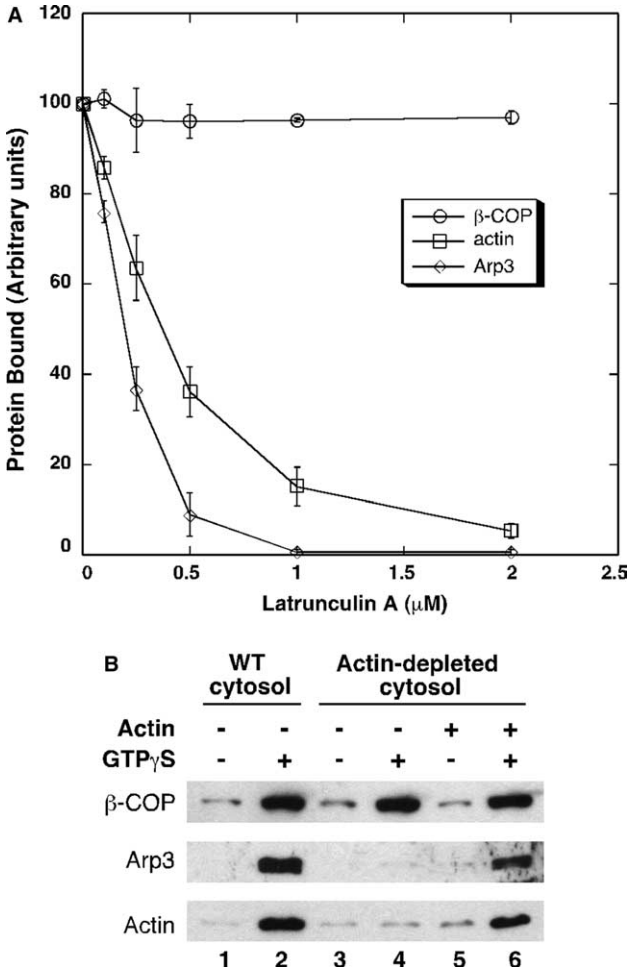

Fig. 3. Arp2/3 recruitment to Golgi membranes requires actin. (A) Golgi-binding assays were carried out in the presence of cytosol and GTPγS plus varying concentrations of latrunculin A. The levels of bound coatomer (β-COP), actin, and Arp2/3 (Arp3) were determined by Western blotting and quantified by densitometry. Shown are the averages from three independent experiments. The bars represent the standard error of the mean. (B) Shown is a Western blot of float-up Golgi-binding assays carried out either with normal bovine-brain cytosol (lanes 1 and 2) or with actin-depleted cytosol (lanes 3–6). The Western blot was probed with antibodies against β-COP, Arp3, and actin as indicated. GTPγS and purified muscle α-actin were added to the incubations where indicated.

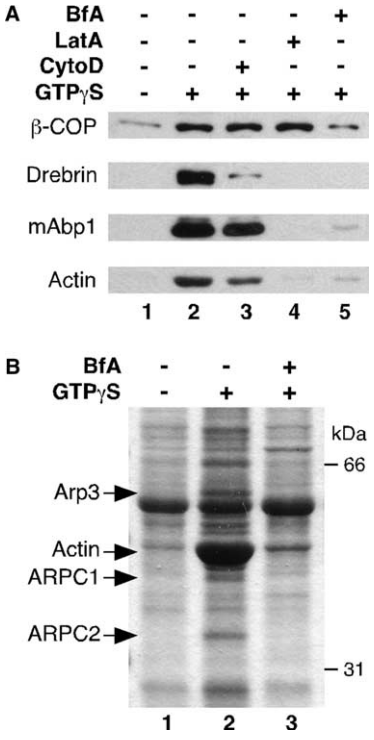

Fig. 4. (A) ARF-regulated actin polymerization can be reconstituted on liposomes. Shown is a Western blot of liposome-binding assays carried out in the presence of bovine-brain cytosol. Brefeldin A (BfA, 200 μM), latrunculin A (LatA, 1 μM), cytochalasin D (CytoD, 20 μg/ml) and GTPγS (20 μM) were added as indicated. The blot was probed with antibodies against β-COP, drebrin, mAbp1, and actin. (B) Arp2/3 binds to liposomes in an ARF-dependent manner. Shown is a Coomassie-blue-stained 12% SDS–PAGE of isolated liposomes following incubations with bovine-brain cytosol. GTPγS and brefeldin A, BfA, were added to the incubations as indicated. The protein bands corresponding to Arp3, ARPC1 and ARPC2 were identified using mass spectrometric analysis of their tryptic fragments (see Section 2). The actin band was identified by comparison with Western blots.

the Golgi membranes were obtained in the presence of cytochalasin D (data not shown). Coatomer binding was unaffected by the toxin treatment (Fig. 3A).

To directly analyze the role of actin in Arp2/3 interactions with the Golgi membranes, we prepared actin-depleted cytosol for use in the Golgi-binding assay. ARF1-dependent Arp2/3 binding and actin assembly on Golgi membranes were inhibited when using actin-depleted cytosol, although ARF1-mediated coatomer assembly on the Golgi membranes was unaffected (Fig. 3B, compare lanes 2 and 4). This inhibition was reversed by the addition of pure skeletal-muscle actin monomers to the reaction (lane 6). Together, these results indicate that binding of the Arp2/3 complex to the Golgi membranes in response to upstream signals requires actin.

3.4. *Cytosolic proteins are sufficient for ARF-mediated actin polymerization*

COPI vesicle assembly can be reconstituted on liposomal membranes that are devoid of membrane proteins [32,33]. We wished to test whether cytosol-derived proteins were also sufficient for ARF/coatomer/Cdc42-dependent actin polymerization. Liposome binding assays confirm that coatomer binds in a GTPγS-dependent manner (Fig. 4A compare lane 1 with lane 2). This binding is sensitive to brefeldin A (lane 5), but resistant to the actin-depolymerizing toxins cytochalasin D (lane 3) and latrunculin A (lane 4). Importantly, we observed that actin and the two actin-binding proteins, drebrin and mAbp1, bind liposomes in a GTPγS-dependent, BFA-sensitive manner (compare lanes 1, 2 and 5). ARF-dependent drebrin association with the liposomes is sensitive to cytochalasin D and latrunculin A (lanes 3 and 4), whereas mAbp1 binding to the liposomes is insensitive to cytochalasin D (lane 3). These findings closely mirror our previous results identifying drebrin and mAbp1 as largely segregated into two different ARF1-dependent actin complexes on the Golgi membranes [3]. From these data, we determined that liposomes faithfully recapitulate several aspects of ARF1-regulated actin polymerization and could take the place of Golgi membranes in the cell-free

reconstitution for identifying candidate proteins that regulate actin dynamics in the early secretory pathway.

Coomassie-blue stained SDS gels from the liposome binding assay revealed that in addition to actin, abundant protein bands of 51, 41 and 34 kDa bound to liposomes only when GTPγS was included in the reactions (Fig. 4B). Recruitment of these proteins to the liposomes was inhibited by the addition of brefeldin A, indicating that the binding is mediated by ARF (compare Fig. 4B, lanes 2 and 3). These three ARF-dependent protein bands were identified as the subunits of the Arp2/3 complex, Arp3 (51 kDa), ARPC1 (41 kDa), and ARPC2 (34 kDa), using mass spectrometry. This result reveals that the Arp2/3 complex is a major ARF1-dependent membrane binding protein. Since Arp2/3 binding and actin polymerization can be reconstituted on liposomes, that are initially devoid of membrane proteins, we conclude that all of the protein machinery necessary for ARF/coatomer-dependent actin polymerization can be recruited from the cytosol during vesicle formation.

4. Discussion

There is increasing evidence indicating the importance of the cytoskeleton for protein trafficking in cells [34]. Specific actin-binding proteins, such as mAbp1 and myosin motor proteins, as well as proteins that regulate actin dynamics, such as Cdc42, N-WASP and Arp2/3, have been implicated in the function of the Golgi apparatus [3,6,12,13]. These proteins are important for the positioning and orientation of the Golgi apparatus as well as for protein trafficking. Since the actin cytoskeleton is involved in numerous cellular processes such as cell motility, organelle distribution, and cell structure formation (i.e., microvilli), it can be challenging to dissect the role and the regulation of actin in the secretory pathway, especially with whole-cell approaches.

For example, we have attempted to examine the effects of mutant ARF1 and Cdc42 on Arp2/3 localization in whole cells. Although we find defects in Arp2/3 localization under these conditions (data not shown), there are also changes in Golgi morphology or global actin dynamics in the cell that make these results difficult to interpret. In this regard, we have now utilized a cell-free reconstitution approach, both on liposomes and on isolated Golgi membranes, that allow ARF-dependent regulation of actin dynamics to be studied in isolation.

We report that Arp2/3 binds to Golgi membranes in response to ARF1 activation. This binding requires the coatomer/Cdc42 complex and very likely the activation of Golgi localized N-WASP. We also find that actin is required for the binding of Arp2/3 to the Golgi membranes. We propose that ARF1 leads to the polymerization of actin by activating Cdc42, N-WASP and Arp2/3. Although coatomer/Cdc42-dependent activation of Arp2/3 accounts for much of the actin that binds to Golgi membranes, our results also indicate that there are additional mechanisms that regulate Golgi actin. In this regard, we reported previously that there are two distinct ARF-dependent actin pools. Only one of these pools, one defined by the binding of the actin-binding protein, mAbp1, is dependent on signaling through coatomer/Cdc42. It will be of interest to clarify which pools of actin are dependent on Arp2/3 and what additional activation mechanisms, for example Scar2, are involved at the Golgi apparatus.

Cdc42 requires the binding interaction with coatomer for its recruitment to the Golgi membranes. Since N-WASP [13] and Arp2/3 also bind to the Golgi apparatus in response to ARF activation, it seems plausible that they also interact with the membranes in a complex with coatomer/Cdc42. Indeed, our results with the p23 peptide in the presence of GTPγS (Fig. 2A) indicate that Cdc42 activation in solution is not sufficient for Arp2/3 localization to the Golgi membranes. Nevertheless, our results show that most, if not all, of the Arp2/3 binding to the membrane requires the presence of actin. Thus, Arp2/3 appears to be activated by coatomer-bound Cdc42 on the membrane and then binds to the membrane via actin filaments.

We have observed that Rac and Cdc42 behave similarly with regard to membrane binding in vitro. There is a preponderance of data supporting the involvement of Cdc42 at the Golgi complex. Nevertheless, a role for Rac cannot be discounted. Additional characterization of Cdc42, Rac and other homologs such as TC10 [35] will be required for a full understanding of the function of Rho-related GTP-binding proteins in the early secretory pathway.

Importantly, our finding that GTPγS-dependent and brefeldin A-sensitive actin polymerization can be reconstituted on liposomes demonstrates that there is not an absolute requirement for integral membrane proteins or preassociated peripheral membrane proteins for actin assembly during vesicle formation. Thus, the coatomer/Cdc42 complex likely recruits all necessary downstream components for this process including N-WASP and Arp2/3 to the Golgi membrane. As the mechanisms for regulating cytoskeletal dynamics in the secretory pathway become clearer, the road will be paved for a better understanding of the function of the cytoskeleton in vesicular trafficking.

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