Architecture of the vimentin cytoskeleton is modified by perturbation of the GTPase ARF1

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Summary

Intermediate filaments are required for proper membrane protein trafficking. However, it remains unclear whether perturbations in vesicular membrane transport result in changes in the architecture of the vimentin cytoskeleton. We find that treatment of cells with Brefeldin A, an inhibitor of specific stages of membrane transport, causes changes in the organization of vimentin filaments. These changes arise from movement of pre-existing filaments. Brefeldin A treatment also leads to alterations in the microtubule cytoskeleton. However, this effect is not observed in cells lacking intermediate filaments, indicating that microtubule bundling is downstream of perturbations in the vimentin cytoskeleton. Brefeldin A-induced changes in vimentin architecture are probably mediated through its effects on ADP-ribosylation factor 1 (ARF1). Expression of a dominant-negative mutant of ARF1 induces BFA-like modifications in vimentin morphology. The BFA-dependent changes in vimentin architecture occurred concurrently with the release of the ARF1-regulated adaptor complexes AP-3 and AP-1 from membranes and adaptor redistribution to vimentin networks. These observations indicate that perturbation of the vesicular membrane transport machinery lead to reciprocal changes in the architecture of vimentin networks.

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Introduction

Vesicular membrane transport is a crucial process required for proper targeting of transmembrane and luminal proteins throughout a series of membrane bound organelles and the plasma membrane (Bonifacino and Glick, 2004; Munro, 2004; Palade, 1975). Several reports indicate that the machinery required for vesicle-mediated transport as well as organelle integrity and positioning are modulated by the intermediate filament cytoskeleton (reviewed by Styers et al., 2005; Toivola et al., 2005). For example, intermediate filaments are required for proper AP-3-mediated membrane protein sorting to lysosomes (Styers et al., 2004), polarized membrane protein delivery in epithelial tissues (Ameen et al., 2001; Toivola et al., 2004), and possibly the function of the vesicle fusion apparatus (Faigle et al., 2000). Furthermore, intermediate filaments also appear to be involved in lysosomal positioning and to interact with the Golgi complex (Gao and Sztul, 2001; Styers et al., 2004). This evidence indicates that the intermediate filament cytoskeleton modulates the maintenance and integrity of membranous compartments in the exocytic and endocytic routes.

One aspect of this interaction that remains largely unexplored, however, is whether perturbations in vesicular membrane transport regulate the architecture of the intermediate filament cytoskeleton. If intermediate filaments and vesicular membrane transport are functionally interconnected, this leads to the prediction that not only should perturbation of intermediate filaments lead to defects in vesicular membrane transport, but that perturbation of transport should have effects on cytoskeletal architecture and/or function. Some data suggest that this could be the case. Overexpression of the resident Golgi and vimentin-interacting protein FTCD leads to bundling of vimentin filaments, probably through a crosslinking mechanism (Faigle et al., 2000; Gao et al., 2002). An understanding of how players in vesicular membrane transport regulate cytoskeletal, and, in particular, intermediate filament architecture, may be particularly important in polarized cell types, such as neurons or polarized epithelia, in which a well-organized intermediate filament cytoskeleton is crucial for proper protein transport and cellular function (Ameen et al., 2001; Perez-Olle et al., 2005; Toivola et al., 2004).

We have undertaken a pharmacological and molecular approach to explore how perturbations in vesicular membrane transport regulate the architecture of the vimentin cytoskeleton. We began by using the fungal metabolite brefeldin A (BFA), which causes perturbations in vesicular membrane transport and organelle integrity, including Golgi fragmentation, changes in ADP ribosylation of cytosolic proteins, and perturbation of the ADP-ribosylation factor 1 (ARF1) GTPase cycle. The latter results in the release of the coat/adaptor-dependent sorting machinery from membranes (Di Girolamo et al., 1995; Donaldson et al., 1992a; Donaldson et al., 1992b; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Ooi et al., 1998; Peyroche et al., 1999; Traub et al., 1993). Here, we demonstrate that treatment with BFA leads to rapid and
reversible changes in the architecture of the intermediate filament cytoskeleton, as well as the microtubule cytoskeleton. However, vimentin filaments appear to be the primary cytoskeletal target of the drug, because microtubule bundling does not occur in the absence of intermediate filaments.

We analyzed the effects of expression of ARF1 mutants that perturb different stages of coat/adaptor membrane recruitment upon vimentin cytoskeletal networks. Expression of a dominant-negative mutation in ARF1 caused similar changes in the vimentin cytoskeleton to those observed in BFA-treated cells, indicating that BFA-induced changes in vimentin networks occurred through its inhibition of ARF1. Finally, because both BFA and dominant-negative ARF1 release AP-3 and AP-1 adaptor complexes from membranes (Ooi et al., 1998; Traub et al., 1993), and AP-3 has previously been shown to interact with vimentin filaments (Styers et al., 2004), we treated cells with BFA and explored the association of these adaptors with vimentin filaments. BFA induced a large increase in binding of the adaptor complexes AP-3 and AP-1 to both insoluble vimentin filaments and soluble vimentin protein. These findings are consistent with the idea that not only do intermediate filaments regulate adaptor-based membrane transport, but also that perturbation of adaptor-based transport mechanisms has reciprocal effects on the architecture of the intermediate filament cytoskeleton.

Results

Brefeldin A alters the architecture of vimentin networks

Previous findings from our laboratory and others suggest that vimentin filaments regulate a subset of adaptor-based vesicular membrane transport events, as well as the position and structure of membranous organelles (Ameen et al., 2001; Faigle et al., 2000; Gao and Sztul, 2001; Styers et al., 2005; Styers et al., 2004; Toivola et al., 2004; Toivola et al., 2005). However, it remains unknown whether perturbations in the vesicular membrane transport machinery and organelle integrity lead to alterations in the architecture and function of vimentin networks. To explore an association between vimentin networks and vesicular membrane transport processes further, we began by assessing the effects of BFA on the vimentin cytoskeleton. We hypothesized that perturbations of the adaptor-based transport machinery and/or the integrity of membranous organelles by BFA might lead to changes in the architecture of vimentin filaments. We initially focused on BFA because drug treatment leads to both Golgi fragmentation and perturbation of the dynamics of adaptor or coat complexes, two processes linked to the intermediate filament cytoskeleton (Donaldson et al., 1992b; Gao and Sztul, 2001; Gao et al., 2002; Helms and Rothman, 1992; Hunziker et al., 1992; Klausner et al., 1992; Orci et al., 1991; Peyroche et al., 1999; Styers et al., 2004). To separate the effects of BFA on vimentin filaments from other cytoskeletal elements, we used SW13 cells as our model. Two clonal variants of this adrenal cortex carcinoma cell line exist, which express either only the intermediate filament vimentin (SW13 v+) or no cytoplasmic intermediate filaments (SW13 v−) (Hedberg and Chen, 1986). In addition, these cells have a characteristically uniform cell shape and cytoskeletal morphology (Fig. 1a), making the assessment of changes in the cytoskeleton clear and easily visualized.

Initially, SW13 v+ cells were treated with 10 μg/ml BFA for 30 minutes or 1 hour at 37°C. Cells were then fixed and stained using monoclonal antibodies directed against vimentin. When compared with control cells, cells treated with BFA exhibited abnormal intermediate filament structures, in which the filaments either retracted to the perinuclear region or formed elongated process-like formations (Fig. 1a). High-magnification epifluorescence images of BFA-treated cells showed that these processes were made up of densely clustered vimentin filaments, which appeared to be aligned in a parallel manner (Fig. 1b). Quantification of cells with altered (either retracted or with process-like formations) vimentin cytoskeletons demonstrated that 23% of cells exhibited altered vimentin cytoskeletons at 30 minutes, and that 96% of cells exhibited altered vimentin cytoskeletons by 1 hour (Fig. 1c). This indicated that treatment with BFA, an inhibitor of specific steps of vesicular membrane transport, led to drastic changes in the architecture of vimentin filaments.

To gain insight into the mechanism of how changes in the vimentin cytoskeleton occurred over time, we used an SW13 v+ cell line stably expressing GFP-vimentin. Morphological changes in vimentin networks could occur either through changes in vimentin polymerization dynamics or by movement of pre-existing vimentin filaments. To differentiate between these possibilities, subunit-exchange dynamics in the presence of BFA were assessed by fluorescence recovery after photobleaching (FRAP). This procedure has previously been used to show that intermediate filaments are dynamic, although subunit exchange occurs more slowly than other cytoskeletal networks (Vikstrom et al., 1992). Cells were either untreated or pretreated with 10 μg/ml BFA for 15 minutes, and then FRAP was performed in the absence or presence of BFA. No changes were observed in the recovery of bleached filaments between treated or untreated cells (Fig. 1d). These data exclude changes in vimentin dynamics induced by BFA and suggest that modifications in vimentin cytoskeleton morphology are due to the movement of preexisting vimentin filaments. To assess this hypothesis, cells were treated with 10 μg/ml BFA and then imaged by spinning disc confocal microscopy for 1 hour (Fig. 1e and supplementary material Movie 1). Both process formation and retraction of vimentin filaments was observed and appeared to result from movement of preexisting individual vimentin filaments. Importantly, the GFP-vimentin processes closely resembled those seen in fixed cells (Fig. 1, compare a,b,e), thus excluding changes in cytoskeletal architecture as an artifact of specimen preparation. No de novo filament formation was observed, consistent with FRAP results. However, in some cells, increased formation of vimentin aggregate or particle-like structures was seen similar to those observed in filament reorganization during mitosis (supplementary material Movie 1) (Franke et al., 1982; Rosevear et al., 1990). These results demonstrate that in both live and fixed specimens, treatment with BFA induces changes in the architecture of the vimentin cytoskeleton, either through retraction to the perinuclear region or through the formation of elongated process-like structures, which form from existing cytoplasmic networks.

Brefeldin A affects the microtubule cytoskeleton in a vimentin-dependent manner

A previous report has indicated that long-term treatment (15 hours or 40 hours) with BFA leads to alterations in the
Fig. 1. Brefeldin A treatment induces changes in the architecture of vimentin networks. (a) SW13 v+ cells were either treated with methanol (Control) or 10 μg/ml BFA for 30 minutes or 1 hour at 37°C. Cells were fixed and stained with monoclonal antibodies directed against vimentin and visualized by epifluorescence microscopy. BFA treatment induces either retraction of vimentin networks to the perinuclear region or the generation of process-like formations when compared with untreated cells. The severity of the phenotype increases with longer treatment. Bars, 20 μm. (b) High magnification (100×) images of cells treated for 30 minutes with 10 μg/ml BFA and 4× magnification of process marked by asterisk (*) show that vimentin processes are made up of densely clustered vimentin filaments. Scale bars are 20 μm. (c) Quantification of results from a. Number of cells showing either retracted vimentin networks or process formation after either 30 minutes or 1 hour of BFA treatment were counted and expressed as a percent of the total number of cells. (d) SW13 v+ cells stably expressing GFP-vimentin were either treated with methanol (Control, ○) or 10 μg/ml BFA (BFA, ▼) for 15 minutes before beginning FRAP. Images were taken every 30 seconds for ~30 minutes. No differences were seen in recovery of the bleached region of the cell between control and BFA-treated cells, indicating that BFA treatment had no effect on subunit-exchange dynamics. (e) Spinning-disc confocal time-lapse imaging of vimentin bundling. SW13 cells stably expressing GFP-vimentin were treated with 10 μg/ml BFA for 1 hour. Both process formation (top panels) and retraction (bottom panels) appeared to arise from preexisting vimentin filaments (see movie 1 in supplementary material).

architecture of both the microtubule and actin cytoskeletons (Alvarez and Sztul, 1999). Since intermediate filaments are known to be tightly associated with these other cytoskeletons through linker molecules and motors (Chang and Goldman, 2004; Coulombe and Wong, 2004; Leung et al., 2002), the effects of BFA on intermediate filaments could be secondary to its effects on microtubules or actin. Therefore, we explored the short-term effects of BFA on these other cytoskeletal elements.

SW13 v+ cells expressing GFP-vimentin were treated with 10 μg/ml BFA and stained using antibodies to α-tubulin to visualize the microtubule cytoskeleton. Similar to vimentin filaments, process-like formations of microtubules were also observed in BFA-treated cells (Fig. 2a). Because intermediate filament networks and microtubule networks are tightly associated, this led us to hypothesize that changes in microtubule architecture were dependent upon the presence of intermediate filaments. To explore this question, we compared microtubule and actin networks in BFA-treated SW13 v− cells, which express no cytoplasmic intermediate filaments, and SW13 v+ cells, which express only vimentin. Treatment of SW13 v+ and v− cells both resulted in Golgi fragmentation as shown by staining with GM130 (supplementary material Fig. S1), indicating that the two cell types did not exhibit differential sensitivity to the drug. Control and BFA-treated cells were also stained for microtubules, using an antibody to α-tubulin, and F-actin, using Rhodamine-conjugated phalloidin (Fig. 2). As shown in Fig. 2b, in SW13 v+ cells treated with BFA, changes in microtubule architecture were observed. However, changes in microtubules were not observed in BFA-treated SW13 v− cells (Fig. 2b). This observation indicates that the presence of intermediate filaments is required for BFA-induced microtubule changes. Although vimentin filaments appear to be the primary
cytoskeletal target of BFA, changes in the architecture of vimentin networks, including its extension, are dependent upon the microtubule cytoskeleton and its associated motors (Helfand et al., 2002; Prahlad et al., 1998). Consistent with these observations, the microtubule-destabilizing drug nocodazole (Ludueña and Roach, 1991) blocked BFA-induced filament rearrangement (data not shown). This suggests that BFA-induced bundling of vimentin filaments is dependent upon the presence of an intact microtubule cytoskeleton. Alterations in actin networks were not observed following BFA treatment either at cell-cell contacts or in membrane ruffles, suggesting that short-term treatment with BFA does not affect the actin cytoskeleton (Fig. 2b). Together, these results suggest that vimentin filaments are the primary cytoskeletal targets of BFA.

Brefeldin A-induced changes in the vimentin cytoskeleton are not caused by overall changes in cellular morphology

It is possible that changes in the architecture of vimentin filaments are an indirect effect of BFA owing to overall changes in cell shape. For example, changes in cell shape could force vimentin filaments into process-like formations, simply due to localized changes in cytoplasmic volume. To test this hypothesis, we treated cells with cytochalasin D, a drug previously shown to inhibit actin polymerization by binding to the barbed end of actin filaments (Cooper, 1987). Actin is required for general changes in cell shape, such as the extension of the leading edge, formation of filopodia, and membrane ruffling (Pollard and Borisy, 2003; Small et al., 1999). Therefore, depolymerization of the actin cytoskeleton should effectively block generalized changes in cell shape. SW13 v+ cells were treated with either 10 µg/ml BFA alone for 30 minutes, 1 µg/ml cytochalasin D alone for 45 minutes, or were pretreated with 1 µg/ml cytochalasin D for 15 minutes followed by the addition of 10 µg/ml BFA in the continued presence of cytochalasin D for 30 minutes. Treatment with cytochalasin D alone resulted in depolymerization of the actin cytoskeleton at the cell cortex and at cell-cell contacts, visualized by staining with antibodies against β-actin (Fig. 3). In addition, it also resulted in a collapse of vimentin filaments and microtubules to the perinuclear region, an observation consistent with the reported association between the actin, microtubule, and intermediate filament cytoskeletons (Chang and Goldman, 2004; Green et al., 1987). However, in cells treated with both cytochalasin D and BFA, process formation and retraction was observed in both cells stained for vimentin or microtubules, similar to that for cells treated with BFA alone (Fig. 3). This indicates that BFA-induced changes in the architecture of the vimentin cytoskeleton are not due to actin-based changes in cell shape.

To assess further whether modifications in vimentin filaments could be due to changes in cell shape, cells were seeded onto coverslips coated with poly(2-hydroxyethyl methacrylate (poly HEMA). Poly HEMA is an organic polymer that behaves as a protein-free non-adhesive substratum matrix. In contrast to Matrigel™, poly HEMA does not support adhesion through cellular adhesion molecules such as integrins (Minett et al., 1984). Since changes in cell adhesion can also trigger changes in cell shape, seeding cells on poly HEMA should prevent changes in cell morphology that are dependent upon cell adhesion. Cells seeded on poly HEMA remained rounded up and not well attached when compared with cells seeded on Matrigel™ (supplementary material Fig. S2). However, treatment with 10 µg/ml BFA for 30 minutes did result in vimentin process formation, visualized by staining with monoclonal antibodies directed against vimentin. This indicates that changes in the architecture of the vimentin cytoskeleton are not due to cell shape changes driven by cytoskeletal associations with the extracellular matrix. Together these results suggest that the BFA-driven
modifications in the vimentin cytoskeleton architecture are due to effects on the intermediate cytoskeleton itself and not a secondary effect of either actin- or extracellular-matrix-based changes in cell morphology (Goldman et al., 1996).

Brefeldin A-induced changes in vimentin cytoskeletal architecture are not due to Golgi fragmentation or ADP-ribosylation

BFA has previously been shown to lead to Golgi fragmentation (Fujiwara et al., 1988) and vimentin networks have been shown to interact with the Golgi complex (Gao and Sztul, 2001; Gao et al., 2002). Therefore, the effects of BFA on vimentin architecture could be due to fragmentation of the Golgi complex. To further explore Golgi fragmentation as a cause of BFA-induced vimentin changes, we used ilimaquinone (IQ), a structurally different drug that exhibits effects similar to BFA in fragmentation of the Golgi complex but differs in the molecular mechanisms underlying this effect (Takizawa et al., 1993; Weigert et al., 1997). In addition to its effects on Golgi fragmentation, IQ has also been shown to result in release of the adaptor COP-I from membranes (Madari et al., 2003; Takizawa et al., 1993). However, the effect of IQ on other BFA-sensitive adaptor complexes has not yet been reported.

If Golgi fragmentation underlies the changes in vimentin architecture, then both BFA and IQ should cause similar effects on the vimentin cytoskeleton. To test this hypothesis, SW13 v+ cells were treated with 10 μg/ml BFA or 40 μM IQ for either 30 minutes or 1 hour. This dose of IQ was previously shown to perturb Golgi dynamics (Weigert et al., 1997). Treatment with BFA and IQ resulted in fragmentation of the Golgi complex as shown by staining with the Golgi marker GM130 (Fig. 4). In addition to and consistent with previous reports, treatment with BFA resulted in the release of both AP-1 and AP-3 from the membrane into the cytoplasm (Fig. 4) (Ooi et al., 1998; Traub et al., 1993). However, treatment with IQ did not result in substantial release of the BFA-sensitive adaptors AP-1 and AP-3 from the membrane (Fig. 4 and supplementary material Fig. S3). Slight changes in AP-1 and AP-3 localization occurred as a result of changes in the localization of Golgi and endosomal membranes (see Fig. 4, compare GM130, AP-1, AP-3 insets). However, Golgi and punctate staining of both AP-1 and AP-3 clearly remained in cells treated with IQ for either 30 minutes or 1 hour (Fig. 4 and data not shown).

The effects of BFA and IQ on the vimentin cytoskeleton also differed. As observed in Fig. 1, treatment with BFA for 30 minutes induced the formation of long vimentin processes and the retraction of the vimentin cytoskeleton. Longer treatment for 1 hour resulted in increases in both the severity and frequency of these phenotypes (Fig. 4). Treatment with IQ did not result in process formation, and instead cells appeared to round up, particularly with longer treatment, although they did not detach from the coverslip (Fig. 4). Because IQ treatment did not result in the formation of vimentin processes, it is unlikely that bundling was due to Golgi fragmentation.

Fig. 3. Depolymerization of actin does not inhibit BFA-induced changes in vimentin networks. SW13 v+ cells were treated in either the absence or presence of 10 μg/ml BFA for 30 minutes, 1 μg/ml cytochalasin D for 45 minutes, or pretreated with 1 μg/ml cytochalasin D for 15 minutes followed by the addition of 10 μg/ml BFA for 30 minutes in the continued presence of cytochalasin D at 37°C. Cells were processed and stained with monoclonal antibodies directed against either vimentin, α-tubulin, or β-actin and visualized by epifluorescence microscopy. Cytochalasin D treatment induced actin depolymerization as shown by β-actin staining. In addition, it also generated retraction of both microtubule and vimentin networks. However, cells treated with both BFA and cytochalasin D exhibited process-like formations of both vimentin and microtubules, indicating that actin was not required for the effects of BFA on vimentin and microtubule networks. Bar, 20 μm.
To exclude the possibility that effects of BFA and IQ on the vimentin cytoskeleton were due to cytotoxicity, we determined whether the effects of these drugs on the vimentin cytoskeleton were reversible. Cells were treated in the absence or presence of 10 μg/ml BFA or 40 μM IQ for either 30 minutes or 1 hour (vimentin 1 h panel only) at 37°C. Cells were then processed for immunofluorescence and stained with monoclonal antibodies directed against vimentin, GM130, the γ subunit of AP-1 or the δ subunit of AP-3. Vimentin staining was visualized by epifluorescence microscopy, and GM130, AP-1 and AP-3 staining was visualized by confocal microscopy. BFA induced changes in vimentin architecture that became more pronounced at 1 hour. IQ did not induce similar changes, although cells were observed to round up, particularly with longer treatments. Both drugs resulted in fragmentation of the Golgi complex, as visualized by GM130 staining. Only BFA resulted in release of the adaptors AP-1 and AP-3 from the membrane. Bars, 20 μm. Insets are of cells indicated by an asterisk (*) and are magnified 3×.

Fig. 4. Brefeldin A and ilimaquinone have differential effects on the vimentin cytoskeleton. SW13 v+ cells were treated in either the absence or presence of 10 μg/ml BFA or 40 μM IQ for either 30 minutes or 1 hour (vimentin 1 h panel only) at 37°C. Cells were then processed for immunofluorescence and stained with monoclonal antibodies directed against vimentin, GM130, the γ subunit of AP-1 or the δ subunit of AP-3. Vimentin staining was visualized by epifluorescence microscopy, and GM130, AP-1 and AP-3 staining was visualized by confocal microscopy. BFA induced changes in vimentin architecture that became more pronounced at 1 hour. IQ did not induce similar changes, although cells were observed to round up, particularly with longer treatments. Both drugs resulted in fragmentation of the Golgi complex, as visualized by GM130 staining. Only BFA resulted in release of the adaptors AP-1 and AP-3 from the membrane. Bars, 20 μm. Insets are of cells indicated by an asterisk (*) and are magnified 3×.

ADP-ribosylated substrate (BARS-50) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Brune and Lapetina, 1995; Di Girolamo et al., 1995). In addition, vimentin and other intermediate filaments have been shown to be substrates of ADP-ribosylation machinery. Changes in filament ADP ribosylation can result in modifications in the architecture of filament networks (Huang et al., 1993; Kaslow et al., 1981; Yuan et al., 1999; Zhou et al., 1996). To understand whether ADP ribosylation was required for BFA-induced vimentin remodeling, cells were treated with 10 μg/ml BFA in the absence or presence of 50 mM nicotinamide. Nicotinamide is a general inhibitor of ADP ribosylation, and this dose is equivalent to 2.5 times the reported IC50 for inhibition of ADP ribosylation of BARS50 (Weigert et al., 1997). As a control to ensure the efficacy of nicotinamide treatment, SW13 v+ cells were treated with 1 mM hydrogen peroxide to induce nuclear poly-ADP ribosylation (Kupper et al., 1996; Schraufstatter et al., 1986). Peroxide-treated cells showed an induction of poly-ADP ribosylation, as shown with a monoclonal antibody to poly-ADP ribose. This signal was specific, because it was not seen in either untreated cells or cells treated with hydrogen peroxide in the presence of catalase, an enzyme that converts hydrogen peroxide to H2O and O2. Nicotinamide effectively blocked poly-ADP ribosylation, demonstrating the efficacy of the inhibitor (supplementary material Fig. S4b). To test whether nicotinamide blocks BFA-induced changes in vimentin filaments, cells were treated with either the vehicle, 10 μg/ml BFA alone, 50 mM nicotinamide alone, or 10 μg/ml BFA in the presence of nicotinamide for 30 minutes (supplementary material Fig. S4a). As previously shown, BFA treatment induced retraction and process formation in the vimentin cytoskeleton. Nicotinamide treatment alone induced minimal changes in the vimentin cytoskeleton. However, nicotinamide treatment did not block BFA-induced changes in vimentin architecture. This indicates that BFA-induced changes in vimentin architecture are not due to ADP ribosylation.

Vimentin cytoskeletal architecture is modified by ARF1 mutants that mimic the effects of BFA Since BFA-induced changes were not due to either Golgi fragmentation or to changes in ADP ribosylation, we next pursued whether these changes might be due to ARF1. ARF1 is a GTPase of the Ras superfamily that regulates GTP-dependent recruitment of coat proteins to membranes to promote vesiculation and budding (Boman and Kahn, 1995;
Crottet et al., 2002; Faundez et al., 1998; Kahn and Gilman, 1986; Ooi et al., 1998; Serafini et al., 1991; Traub et al., 1993; Zhu et al., 1998). Previous work has shown that the molecular targets of BFA are members of a Sec7-domain-containing family of guanine nucleotide-exchange factors (ARF-GEFs) that catalyze GTP exchange of ARF family proteins (Helms and Rothman, 1992; Morinaga et al., 1999; Morinaga et al., 1997; Morinaga et al., 1996; Peyroche et al., 1999; Robineau et al., 2000; Sata et al., 1998). BFA acts to stabilize a transient complex between the Sec7 domain and the ARF GTPase, which is trapped in the GDP-bound conformation (Robineau et al., 2000). Stabilization of this complex then leads to a net cellular decrease in ARF-GTP availability.

To test whether ARF1 mediates changes in vimentin architecture, SW13 v+ cells were transiently transfected with either wild-type ARF1 fused to GFP, or with two mutant forms of ARF1 fused to GFP. ARF1-T31N has previously been shown to be a dominant-negative mutation causing ARF1 to exist primarily in a GDP-bound, or non-membrane-associated state. This mutant mimics the effects of BFA on coats (Dascher and Balch, 1994). ARF1-Q71L is a dominant-active mutation that restricts ARF1 primarily to a GTP-bound state, resulting in constant activation and retention of coats on membranes (Dascher and Balch, 1994). As previously reported (Dascher and Balch, 1994), control cells stained for the Golgi marker GM130 demonstrated a fragmented in Golgi in the presence of ARF1-T31N, but not in the presence of the wild type or the Q71L mutation (supplementary material Fig. S5). In addition, staining for the adaptor protein complex AP-3, whose membrane recruitment has been shown to be regulated by ARF1 (Ooi et al., 1998), demonstrated that the T31N mutation also resulted in release of the adaptor complex AP-3 from the membrane, similar to BFA treatment (compare Fig. 6b with Fig. 4). Importantly, when ARF1-transfected cells were stained using a monoclonal antibody against vimentin, 62% of cells transfected with the T31N mutation exhibited altered vimentin cytoskeletons, compared with approximately 10% of cells transfected with the wild-type protein (Fig. 6a). Approximately 29% of cells expressing the dominant-active ARF1-Q71L mutation also exhibited altered cytoskeletons; however, it is unclear whether this slight effect is due to changes in the dynamics of ARF1 effectors or to cytotoxic effects of expression of this mutation. However, the significant changes in the cytoskeleton induced by expression of ARF-T31N indicate that the effects of BFA on the vimentin cytoskeleton are probably mediated through ARF1 and its downstream effectors, such as the adaptor-based sorting machinery.

**Brefeldin A treatment increases the association between adaptor complexes and vimentin**

Since a dominant-negative mutation in ARF1 led to a similar effect on the vimentin cytoskeleton as treatment with BFA, we next explored whether downstream effectors of ARF1 may be involved in changing the architecture of vimentin filaments. We have previously noted that treatment with BFA causes the release of the ARF1-regulated adaptors AP-3 and AP-1 from the membrane, whereas in the presence of IQ, which did not cause changes in vimentin architecture, these adaptors are retained on the membrane (Fig. 4). In addition, we have previously shown that the adaptor complex AP-3 interacts directly with vimentin (Styers et al., 2004). Therefore, the effects of BFA on vimentin filaments could be mediated through the release of adaptor complexes from the membrane. This hypothesis leads to the prediction that BFA should induce binding of adaptor complexes to vimentin filaments, and that adaptors should be found on vimentin processes.

As shown previously, soluble vimentin binds specifically and directly to the β3 subunit of the AP-3 complex (Fig. 7a) (Styers et al., 2004). SDS polyacrylamide gel electrophoresis of AP-3 immunoprecipitated from rat brain cytosol results in the resolution of the four subunits of AP-3 by their characteristic molecular masses. When nitrocellulose membranes containing the resolved AP-3 subunits are probed with purified vimentin, antibodies to vimentin bind specifically to a band that co-migrates with the β3 subunit of AP-3, not seen in control lanes lacking cytosol or using control antibodies (Fig. 7a) (Styers et al., 2004). This indicates that vimentin directly interacts with the β3 subunit of AP-3.

To explore whether there was a preferential association of AP-3 with vimentin following BFA treatment, we began by staining control and BFA-treated SW13 cells with antibodies against vimentin and AP-3. We found that upon BFA treatment, AP-3 immunoreactivity partially localized to vimentin processes (Fig. 7b). To quantify the amount of adaptors
associated with vimentin filaments biochemically, we pre-
treated SW13 vimentin positive (v+) or vimentin negative (v–)
cells with 10 μg/ml BFA for 30 minutes at 37°C. Both BFA-
treated and untreated cells were then extracted with 1% Triton
X-100, and adaptor content in the insoluble cytoskeletal pool
was assayed by immunoblot. As previously reported, we saw
a preferential retention of the adaptor complex AP-3 associated
with insoluble intermediate filament networks (Fig. 7c,
compare lanes 1 and 2) (Styers et al., 2004), despite equal
adaptor content in these cell lines (Fig. 7c, lanes 5-6). In
addition, we also found a slight (1.6-fold) increase in retention
of another BFA-sensitive adaptor complex, AP-1. Interestingly,
treatment with BFA led to a substantial increase in the
association of both adaptor complexes with insoluble vimentin
networks (Fig. 7c, compare lanes 2 and 4). Specifically, BFA
treatment led to a 3.8-fold increase in the association of AP-3
with vimentin networks, and a 3.7-fold increase in the
association of AP-1 with vimentin networks (Fig. 7d).

We confirmed the observation that BFA triggers a
preferential association of adaptor complexes with vimentin by
immunoprecipitation from detergent-soluble extracts. Briefly,
SW13 v+ and v– cells were pretreated or not with BFA.
Immunoprecipitations were then performed from detergent-
soluble extracts using antibodies against the δ subunit of AP-
3 or the γ subunit of AP-1. Immunoprecipitates were assayed
for the presence of vimentin by western blot. Although
vimentin could not be reliably detected in immunoprecipitates
from untreated cells (Fig. 7e, lanes 1-2), treatment with BFA
led to an increase in the association of vimentin with both AP-
3 and AP-1 (Fig. 7e, lanes 3-4). This interaction was specific,
as it was not seen in control immunoprecipitations using a non-
specific antibody (Fig. 7e HA). Therefore, together, these data
indicate that BFA treatment induces a preferential association
of the adaptor complexes AP-1 and AP-3 with the vimentin
intermediate-filament network. These results are consistent
with the idea that BFA-induced release of ARF effectors from
membranes triggers changes in the architecture of the vimentin
cytoskeleton.

![Vimentin cytoskeletal architecture is modified by the ARF1 dominant-negative mutant T31N. SW13 v+ cells were transiently transfected with either wild-type ARF1, ARF1-T31N or ARF1-Q71L fused to GFP. Cells were then fixed and processed for immunofluorescence and stained with antibodies directed against either vimentin (a) or the δ subunit of AP-3 (b). (a) ARF1-T31N induced process formation in vimentin networks similar to BFA-treated cells. Approximately 66% of cells expressing ARF1-T31N showed either process formation or retraction (quantification on right). The wild type and Q71L mutants did not show the same effects as T31N on vimentin networks. (b) ARF1-T31N, but not the wild type or Q71L mutants, induced release of the adaptor AP-3 from the membrane. Bars, 10 μm.](attachment:image.png)
Discussion
Here, we provide evidence that pharmacological (BFA) or molecular (ARF1-T31N) perturbation of the vesicular membrane transport machinery leads to rapid and reversible alterations in the morphology of the vimentin intermediate-filament cytoskeleton. This work, together with our previous report that intermediate filaments regulate adaptor-dependent membrane protein sorting to lysosomes (Styers et al., 2004), suggests that intermediate filaments and the machinery for vesicle biogenesis are intimately connected and perturbations in each lead to effects on the other.

We have demonstrated that in SW13 cells, perturbation of vesicular membrane transport using the drug BFA leads to a rapid reorganization of the vimentin cytoskeleton, which either retracts or forms long processes of parallel vimentin filaments. This reorganization is caused by movement of pre-existing vimentin filaments because BFA does not induce changes in the rate of subunit exchange demonstrated by FRAP and as revealed by live imaging of the process. Although BFA treatment also leads to similar changes in microtubules, microtubules do not appear to be the primary target of the drug because microtubule bundling is not apparent in cells lacking intermediate filaments. Moreover, changes in the cytoskeleton do not appear to be secondary to changes in cell shape because depolymerization of the actin cytoskeleton or seeding cells on a non-adhesive matrix does not prevent changes in vimentin morphology.

Because BFA has multiple effects on membrane trafficking and organelle integrity, we next tried to understand the mechanism by which BFA affects vimentin filaments. Because vimentin has previously been shown to associate with Golgi fragments and BFA treatment leads to Golgi fragmentation, we treated cells with IQ, a drug that is structurally different from BFA but causes similar effects on fragmentation of the Golgi complex (Takizawa et al., 1993; Weigert et al., 1997). This was particularly important because fragmentation of the Golgi complex (Takizawa et al., 1993; Weigert et al., 1997). This was particularly important because fragmentation of the Golgi
complex by overexpression of the protein formamidyl transferase cyclodeaminase (FTCD) has been shown to lead to bundling of vimentin filaments (Gao et al., 2002). IQ treatment did not result in bundling of either vimentin filaments or microtubules. Since both BFA and IQ treatment leads to fragmentation of the Golgi complex, it is unlikely that Golgi fragmentation is the cause of vimentin bundling. BFA is also known to induce ADP ribosylation of cytosolic proteins (Brune and Lapetina, 1995; Di Girolamo et al., 1995), and vimentin has previously been shown to be a substrate for ADP ribosylation (Coburn et al., 1989; Huang et al., 1993; Kaslow et al., 1981; Yuan et al., 1999; Zhou et al., 1996). Therefore, we treated cells with BFA in the presence of the general ADP-ribosylation inhibitor, nicotinamide. Nicotinamide treatment did not block BFA-induced changes in the architecture of vimentin filaments, suggesting that changes were not mediated by ADP ribosylation.

ARF family proteins are the best-characterized targets of BFA. BFA has previously been shown to stabilize a complex between Sec7 ARF-GEF family members and ARF proteins, resulting in a net loss of ARF-GTP (Donaldson et al., 1992b; Morinaga et al., 1998; Peyroche et al., 1999; Robineau et al., 2000). Therefore, mutations in ARF that cause the protein to be predominantly found in its GDP-bound (or inactive, T31N) state mimic the effects of BFA (Dascher and Balch, 1994). We found that only ARF1-T31N induced changes in the vimentin cytoskeleton, similar to those observed in BFA-treated cells. These results indicate that ARF1 or ARF1 effectors mediate the changes in the architecture of the vimentin cytoskeleton.

We have previously identified a direct interaction between the adaptor complex AP-3 and vimentin (Styers et al., 2004). Because both BFA and ARF-T31N cause the release of AP-3 (as well as the related adaptor AP-1) from membranes (Ooi et al., 1998; Traub et al., 1993), we elected to explore the association of the adaptor complexes with vimentin filaments following BFA treatment. BFA treatment led to an increased association of adaptors with both insoluble vimentin filaments and soluble vimentin protein. In addition, vimentin processes labeled with AP-3 immunoreactivity following BFA treatment. These data are consistent with the idea that BFA-induced release of adaptors from membrane pools may lead to changes in the architecture of vimentin filaments by a direct interaction.

BFA may mediate changes in the morphology of the vimentin cytoskeleton through several potential mechanisms. ARF1 effectors, such as adaptors, released into the cytosol by BFA treatment may act to crosslink vimentin filaments. This mechanism is similar to that proposed for FTCD (Gao et al., 2002). However, this hypothesis requires either the presence of multiple binding sites for filaments on each adaptor or for adaptors to form multimers. Unlike FTCD, adaptors are not known to exist as multimers. An alternative mechanism is that adaptor release recruits factors required for bundling to vimentin filaments or displaces factors from filaments that prevent bundling. This is an interesting possibility because the adaptor AP-1 has been shown to interact with the microtubule-based motor Kif13a (Nakagawa et al., 2000). In addition, although AP-3 has not been shown to interact directly with motor proteins, multiple motors have been found to be associated with AP-3 derived vesicles (Salazar et al., 2005). Increased association of adaptors with vimentin filaments may cause recruitment of microtubule-based motors to the filaments, leading to microtubule-based process formation and retraction. Finally, a known (or unknown) BFA target, downstream of ARF, could directly modulate changes in filament architecture. An independent role of ARF is difficult to distinguish from adaptor release, however, since adaptor release from membranes is ARF dependent (Boehm et al., 2001; Hirst et al., 1999; Ooi et al., 1998; Stamnes and Rothman, 1993; Traub et al., 1993).

In summary, we have identified an unsuspected effect of perturbation of vesicular membrane transport by BFA through ARF1 on vimentin filament morphology. In addition, we have found that BFA-induced changes in vimentin morphology also lead to changes in the microtubule cytoskeleton. Although the mechanism of these changes in filament architecture needs further exploration, this observation suggests that BFA treatment leads to effects not only on membrane trafficking, but also on the cytoskeleton. The effects on the cytoskeleton are important because they could underlie some of the later effects observed in response to BFA, including changes in organelle morphology and tubulation of the trans-Golgi network and endosomes (Lippincott-Schwartz et al., 1991). In addition, this report also provides further support for the notion that vimentin filaments are closely tied to the vesiculation and sorting machinery, strengthening the hypothesis that bidirectional functional relationships exist between intermediate filaments and adaptor complexes.

Materials and Methods

Cell culture and transfection

SW13 cells were a gift of Dr Robert Evans (Holwell et al., 1997; Sarria et al., 1992). SW13+ cell line stably expressing GFP vimentin was a gift of Dr R. Liem (Columbia University, NY). These cell lines were cultured as previously described (Holwell et al., 1997; Sarria et al., 1992). Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Arf1-GFP constructs were a gift of G. Romero (University of Pittsburgh, PA, USA) and are described (Vanueveen et al., 1998).

Antibodies

Monoclonals used were: anti-tubulin DM1A, anti-vimentin V9, and anti-fl-actin AC-15 (Sigma, St Louis, MO), anti-GM130 and anti-z-actin (BD Transduction Laboratories, San Jose, CA), anti-poly-ADP-ribose 10H (BD Pharimingen, San Diego, CA), anti-HA 12CA5 (a gift from Dr Y. Altschuler, Tel Aviv University, Tel Aviv, Israel), and anti-delta SA4 (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA). Polyclonal anti-hamster vimentin 314 was a gift of Dr Robert Goldman, Northwestern University, Chicago, IL. AP-3 polyclonal antibodies directed against the β3 subunit were previously described (Faindez and Kelly, 2000).

Drug treatments

Unless otherwise specified, SW13 cells were seeded on glass coverslips coated with Matrigel™ (BD Biosciences, San Jose, CA). Poly HEMA (Sigma)-coated coverslips were prepared using a solution of 6 mg/ml in 95% ethanol which was added to coverslips at 5.0 ml/cm² and allowed to evaporate overnight before seeding cells. Drug treatments were performed in media buffered with 20 mM HEPES at the following doses: Brefeldin A (Epicentre Biotechnologies, Madison, WI), 10 μg/ml final concentration (10 mg/ml stock prepared in methanol); ilimaquinone (Sigma), 40 μM final concentration (20 mM stock prepared in DMSO); cytochalasin D (Sigma), 1 μg/ml final concentration (1 mg/ml stock prepared in ethanol); nocodazole (Calbiochem, San Diego, CA), 10 μM final concentration (5 mM stock prepared in DMSO); and nicotinamide (Fisher, Hampton, NH), 50 mM final concentration (5 mM stock prepared in H2O). Following treatment, cells were washed three times in HEPES-buffered medium and either allowed to recover for the allotted times or fixed and processed for immunofluorescence.

For the ADP ribosylation control experiment, SW13 cells were treated with 1 mM H2O2 (30% Stock Solution; Fisher) in DPBS with Ca2+/Mg2+ (Cellgro, Herndon, VA) for 10 minutes in the absence or presence of 20 μM bovine catalase (Calbiochem) or 50 mM nicotinamide. Cells were then fixed and processed for immunofluorescence as described in the manufacturer’s protocol for the anti-poly-ADP-ribose antibody (BD Pharimingen).
Microscopy

Cells were fixed and processed for immunofluorescence as described (Faundez et al., 1997). The only exception was for cells stained using antibodies to the & subunit of AP-3 (SA4). These cells were fixed in 4% paraformaldehyde for 10 minutes at 4°C, which was then removed and fresh 4% paraformaldehyde was added for an additional 20 minutes. Secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse, Alexa Fluor 568 goat anti-mouse, and Alexa Fluor 568 goat anti-rabbit (Molecular Probes, Eugene, OR). F-actin was visualized by staining with Rhodamine-conjugated phalloidin according to the manufacturer’s protocol (Molecular Probes).

Immunoprecipitation, extraction and vimentin overlay assay

SW13 cells were seeded in 6-well plates in DMEM (CellGro) buffered with 1X RPMI 1640 (Gibco/BRL, Gaithersburg, MD). Cells were allowed to reach 80% confluence, washed with DPBS and serum free DMEM (CellGro) buffered with 20 mM HEPES and containing 10 ng/ml of BFA. Soluble proteins were extracted as described (Helfand et al., 1997). The only exception is for cells stained using antibodies to the vimentin intermediate filament cytoskeleton by a Golgi protein. [comment].

Results

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