Aluminum Fluoride Stimulates Surface Protrusions in Cells Overexpressing the ARF6 GTPase

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Abstract. To study the effector function of the ADPribosylation factor (ARF) 6 GTP-binding protein, we transfected HeLa cells with wild-type, epitope-tagged ARF6. Previously shown to indirectly activate the ARF1 GTPase, aluminum fluoride (AIF) treatment of ARF6-transfected cells resulted in a redistribution of both ARF6 and actin to discrete sites on the plasma membrane, which became increasingly protrusive over time. The effects of AIF were reversible, specific to cells transfected with wild-type ARF6, and resembled the cellular protrusions observed in cells expressing the GTPase defective mutant of ARF6. Importantly, the protrusions observed in cells transfected with ARF6 were distinct from the enhanced stress fibers and membrane ruffles observed in cells transfected with RhoA and Rac1, respectively. In cells forming protrusions, there was an apparent stimulation of macropinocytosis and membrane recycling within the protrusive structures. In contrast, no block in transferrin uptake or alteration of the distribution of clathrin AP-2 complexes was detected in these cells. The AIF-induced, ARF6-dependent formation of protrusive structures was blocked by cytochalasin D and inhibitors of the lipoxygenase pathway. These observations support a novel role for the ARF6 GTPase in modeling the plasma membrane and underlying cytoskeleton.

The actin cytoskeleton is a dynamic, structural component of cells that plays a key role in determining cell shape, motility, and cytokinesis. The assembly of actin filaments in cells is regulated tightly and, depending upon the association with numerous proteins, results in a distinctive cellular morphology (Bretscher, 1991; Condeelis, 1993; Stossel, 1993). Two major types of actin arrangements are found in most mammalian cells. Actin stress fibers are composed of contractile bundles of actin filaments, extending across the cell and anchored to the plasma membrane at focal adhesions. Additionally, a mesh of cortical actin filaments is responsible, through assembly and disassembly, for forming the transient ruffles and protrusions of cells (Harris, 1989). Changes in cortical actin can be initiated through various signal transduction pathways and, as a consequence, result in increased cell motility and chemotaxis (Condeelis, 1993; Stossel, 1993; Jamney, 1994). The mechanisms whereby extracellular signals are transduced into specific changes in the actin cytoskeleton remain to be determined.

An important breakthrough in understanding how the actin cytoskeleton could be regulated was the observation that Rho-related GTP-binding proteins of the Ras superfamily appear to regulate the formation of these structures (Hall, 1994; Takai et al., 1995). In Swiss 3T3 cells, evidence from microinjection of wild-type and mutant proteins suggests that RhoA regulates stress fiber formation (Ridley and Hall, 1992), while Rac1 regulates membrane ruffling or lamellopodia (Ridley et al., 1992). In Saccharomyces cerevisiae, changes in actin distribution and assembly are important during vegetative budding, and a series of Rho-related GTPases, including Rho and Cdc42 (Adams et al., 1990), have been implicated in bud site selection. Recently, the mammalian homologue of Cdc42 has been identified and implicated in the extension of microspikes or filopodia. In addition, Cdc42 may in turn activate Rac1, which can then activate RhoA as well (Kozma et al., 1995; Nobes and Hall, 1995). Overall, the Rho-related GTPases can be activated through plasma membrane signaling pathways, including receptor-tyrosine kinases as well as G protein-coupled receptors.

The ADP-ribosylation factor (ARF) family of proteins comprises another group of the Ras superfamily of small GTPases, found in all eukaryotes from S. cerevisiae to man (Kahn et al., 1991). Named for its role as a cofactor in the

1. Abbreviations used in this paper: AIF, aluminum fluoride; ARF, ADP-ribosylation factor; 4BPB, 4-bromophenylacyl bromide; FAK, focal adhesion kinase; FD, fluorescein dextran; HA, influenza hemagglutinin; LCA, lens culinaris agglutinin; NDGA, nordihydroguaretic acid; PLA2, phospholipase A2; PLD, phospholipase D.
cholera toxin-catalyzed ADP-ribosylation of Go (Kahn and Gilman, 1986). ARFs also have recently been shown to stimulate the activity of phospholipase D in vitro assays (Brown et al., 1993; Cockcroft et al., 1994), raising speculation that ARFs may exert their effects by altering phospholipid metabolism (Kahn et al., 1993; Liscovitch and Cantley, 1995). However, such effects have not been directly demonstrated in vivo. The ARF1 protein is abundant in cells and has been thoroughly studied in whole cells and in cell-free biochemical assays (Donaldson and Klausner, 1994). It is required for the binding of coatamer (COPI) protein to Golgi membranes and is necessary for the maintenance of Golgi complex integrity and transport along the secretory pathway (Donaldson and Klausner, 1994; Rothman, 1994). Less is known about the other mammalian ARF proteins, although they appear to be expressed in all cell and tissue types (Tsushiya et al., 1991).

To begin to define the functions of the other ARF proteins, we have taken the approach, beginning with the ARF6 protein, of analyzing the localization and phenotypes of transiently expressed, epitope-tagged ARF proteins and their mutants in mammalian cells (Peters et al., 1995). The validity of this approach has been demonstrated for the ARF1 protein in that overexpression of ARF1 and its mutants in cells recreates within the cell the phenotypes observed in in vitro biochemical assays (for review see Donaldson and Klausner, 1994). Using this approach, we found that the wild-type ARF6 protein is distributed along the cytoplasmic face of the plasma membrane and associated with an internal, tubulovesicular compartment. A mutant of ARF6 predicted to be defective in GTP binding (T27N), and hence predominantly in the inactive, GDP state, is associated almost exclusively with the internal endosome-like structures. Another mutant of ARF6 predicted to be defective in GTP hydrolysis (Q67L), and thus predominantly in the active, GTP state, is confined to the plasma membrane. Whereas the morphology of cells expressing wild-type ARF6 protein appears normal, the morphology of cells expressing mutant forms of ARF6 is altered. Cells expressing T27N exhibit an accumulation of ARF6/T27N-positive tubular-endosomal structures; cells expressing the Q67L mutant exhibit an elaboration of plasma membrane folds at peripheral edges of these cells (Peters et al., 1995).

To identify the function of ARF6 and to study the regulation of its GTP cycle in cells, we turned to approaches found to be useful in studying the ARF1 protein. Studies with the wild-type ARF1 protein were facilitated by the ability to shift the ARF1-GTP cycle in vivo and in vitro with pharmacologic reagents (Donaldson and Klausner, 1994). Whereas GTP exchange onto ARF1 is specifically inhibited by brefeldin A (Donaldson et al., 1992; Helms and Rothman, 1992; Randazzo et al., 1993), GTP hydrolysis can be inhibited by loading the ARF1 protein with GTPγS, a poorly hydrolyzable analog of GTP, or by treatment of intact cells with aluminum fluoride (AlF3) (Melaconnon et al., 1987; Donaldson et al., 1992a; Robinson and Kreis, 1992; Traub et al., 1993). Although AlF3 is a known activator of trimeric G proteins, it has not been shown to activate directly any low molecular weight GTPase, including ARF (Kahn, 1991). Nevertheless, we have demonstrated that in the presence of Golgi membranes, AlF3 acts to protect ARF1-GTP from GTP hydrolysis, perhaps by inhibiting an ARF-GTPase-activating protein, thus inducing stable COPI binding to membranes (Finazzi et al., 1994).

Here we report that treatment of cells overexpressing wild-type ARF6 with aluminum fluoride results in the formation of peripheral plasma membrane protrusions. These protrusive structures contain ARF6, actin, and membranous folds, and are dynamic structures forming and disassembling over a period of minutes. The protrusions induced by AlF3 resemble those observed in cells expressing the GTPase-deficient ARF6 mutant, Q67L. The AlF3-induced response is specific for cells transfected with the wild-type ARF6 protein, is not observed in cells overexpressing ARF1, and is distinct from the actin rearrangements observed in cells overexpressing Rac1 or RhoA proteins. The AlF3-induced protrusive structures were not formed in the presence of cytochalasin D or in the presence of compounds known to inhibit the lipoxigenase branch of arachidonic acid metabolism. Coupled to the dynamic formation of protrusions is an apparent stimulation of membrane turnover via macropinocytosis. Taken together, these results suggest that ARF6, like the Rho-related GTPases, can regulate specific changes in plasma membrane architecture.

Materials and Methods

Reagents, Cells, and Antibodies

Iron-saturated transferrin was obtained from Miles Scientific Co. (Naperville, IL), and lysine-fixable fluorescein dextran (mol wt 10,000) was purchased from Molecular Probes, Inc. (Eugene, OR). Rhodamine isothiocyanate-lens culinaris agglutinin (LCA) was purchased from Vector Labs, Inc. (Burlingame, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

HeLa and COS-7 cells were grown at 37°C in DME supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml gentamicin sulfate.

A mouse antibody (12CA5) against the influenza hemagglutinin (HA) epitope was purchased from BAbCo (Berkeley, CA), and a mouse antibody to the EE epitope (MEYMPMEHM) was the gift of C.J. DeR (University of North Carolina, Chapel Hill). The following mouse antibodies were obtained from Sigma Chemical Co.: anti-gelsolin, anti-talin, anti-human vinculin. Mouse antibodies to focal adhesion kinase (Fak) and cortactin were kindly provided by T. Parsons (University of Virginia Medical Center, Charlottesville) (Kanner et al., 1990). A mouse mAb, AP6, directed against the α subunit of clathrin AP2 (Chin et al., 1988) was kindly provided by F. Brodsky (University of California at San Francisco). Rabbit anti-human transferrin and mouse anti-human transferrin receptor (B3/25) serum were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Fluorescein- or rhodamine-labeled donkey anti-mouse IgG and donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Rabbit polyclonal antisera was raised against a peptide, residues 94-110 of the ARF6 protein. The antisera specifically recognizes the ARF6 protein in transfected cells; the labeling was not observed by immunofluorescence or immunoblotting in the presence of the immunizing peptide.

Plasmids and Transient Transfections

The cDNAs encoding human Rac1 and RhoA (0.6 kb) were excised from the original pZIP-Neo vectors (obtained from R. Khorosvani-Far and C.J. Der, University of North Carolina, Chapel Hill) with BamHI and subcloned into the same modified pCDL-SRκ expression vector (termed pXS; Takebe et al., 1988) used to express either wild-type or mutant ARF6. These Rac1 and Rho A cDNAs contained the nine-amino acid epitope tag (MEYMPMEHM; termed EE) (Hawkins et al., 1995).

HeLa and COS-7 cells grown on glass coverslips were transfected using the calcium-phosphate method as previously described (Bonifacino et al., 1989).
Cells in 10-cm dishes were transfected with 20 μg of plasmid DNA in 10 ml of medium. For six-well dishes, each well received 5 μg of plasmid in 3 ml of medium unless stated otherwise. 36–48 h after transfection, cells were treated as described, fixed with 2% formaldehyde in PBS for 10 min at room temperature, washed with PBS, and incubated with primary antibodies diluted in buffer A (10% FBS and 0.02% azide in PBS) containing 0.2% saponin for 1 h at room temperature. Cells were washed (3 times, 5 min each) with buffer A and incubated with fluorescently labeled secondary antibodies (in buffer A/0.2% saponin) for 1 h, washed again, and mounted on glass slides.

**Internalization of Transferrin and Fluid-phase Uptake of Fluorescein-dextran**

For transferrin uptake, transfected cells were rinsed briefly three times with 0.5% BSA in DME, and then incubated in the same medium for 30 min at 37°C. The cells were then incubated with 30 μg/ml iron-saturated human transferrin either in the presence or absence of 30 mM NaF and 50 μM AlCl₃ for 30 min at 37°C. The cells were then rinsed twice quickly with complete medium (containing 10% FBS), fixed, and processed for indirect immunofluorescence as above (Peters et al., 1995).

To monitor fluid-phase endocytosis, we used fluorescein dextran (mol wt 10,000) as a tracer. After various treatments, cells were incubated at 37°C with 3 mg/ml fluorescein dextran (FD) in complete medium supplemented with 25 mg/ml unlabeled dextran (to reduce nonspecific binding of FD to the cell surface). The coverslips were quickly rinsed to remove free FD by immersing several times into complete medium, and then the cells were fixed with 2% formaldehyde in PBS for 10 min at room temperature. The cells were rinsed for 5 min in buffer A, and then the cell surface was labeled with 0.5 mg/ml rhodamine-LCA (Vector Labs, Inc.) in buffer in the absence of saponin for 30 min. Coverslips were rinsed three times (5 min each) with buffer A and once with PBS, and then mounted onto slides with fluoromount G (Southern Biotechnologies, Birmingham, AL).

**Results**

To begin to study ARF6 function in cells, we looked for reagents that might alter the distribution of wild-type ARF6 in transfected cells. We took advantage of the observation made previously by us (Donaldson et al., 1992a; Finazzi et al., 1994) and others (Melaconv et al., 1987; Robinson and Kreis, 1992) that for the ARF1 protein and its effector function (assembly of coat proteins on Golgi membranes), treatment of intact cells with AIF, a mixture of AlCl₃, and NaF, could mimic the effects of GTP/SG treatment of permeabilized cells, and thus reveal the ARF1-GTP effector function. We therefore tested the effect of AIF treatment on the distribution of wild-type ARF6 protein in transfected cells.

**AIF Induces Surface Protrusions in ARF6-transfected Cells**

We transfected HeLa cells with a plasmid encoding the wild-type influenza hemagglutinin (HA)-tagged ARF6 protein, and then assessed by immunofluorescence the distribution of the expressed ARF6 protein with an antibody to the HA epitope (12CA5), and the structure of the actin cytoskeleton with rhodamine-labeled phalloidin. The ARF6 protein was distributed along the plasma membrane, in microvilli and in focal contact sites at the peripheral edges of these polygonal-shaped cells. Additionally, ARF6 was observed in punctate, endosomal structures. 5 min after addition of AIF, there was an apparent increase in ARF6-HA at the peripheral locations, which became more protrusive with time (Fig. 1). At 10 min, in addition to increased ARF6-HA labeling, fibrilar, HA-labeled structures oriented towards these protrusions were also observed. At this time, increased phalloidin staining became apparent in these protrusive sites. After a 30-min incubation in AIF, elaborate structures were observed, enriched in ARF6 and actin filaments. With further incubation (120 min), these cell surface protrusions became more exaggerated or sometimes broadened into lamellar extensions; at these latter times, the distribution of the ARF6-HA became more diffuse. Imaging of live HeLa cells with video microscopy demonstrated that the membrane protrusions formed during the first 60 min of AIF treatment were dynamic structures; active membrane ruffling and protrusions could be observed forming and retracting at a given site over a period of 5–10 min. After 60-min AIF treatment, the protrusive activity became less dynamic (not shown). The protrusions were not observed in all transfected cells, but at any given time could be observed in >50% of the transfected cells. The presence of the HA epitope on ARF6 was not responsible for this response to AIF since transfection of cells with untagged ARF6 protein elicited the same response (not shown).

The effects observed with AIF treatment were fully reversible. When cells were treated with AIF for 30 min, washed, and incubated in fresh media, the protrusions were no longer evident, and both ARF6 and actin returned to the initial, untreated distributions within 30 min (not shown). Protrusion formation required that the cells be transfected with the wild-type ARF6 plasmid, and thus overexpressing the ARF6 protein, since they were not observed in untransfected HeLa cells. However, in COS cells transfected with ARF6, which also demonstrated the same response to AIF treatment (data not shown), protrusions were observed after AIF treatment in some cells, despite an inability to detect the transfected protein with the antibody to HA. Interestingly, by Northern blot analysis, COS cells appear to have higher endogenous ARF6 mRNA levels than other tissue-culture cells (Tsuchiya et al., 1991), and we have detected higher amounts of endogenous ARF6 protein in COS as compared with HeLa cells (data not shown). These observations suggest that lower expression levels of the transfected ARF6, below the level of detection with the antibody, and possibly endogenous levels in some cell types may be sufficient to observe this response.

We assessed by immunofluorescence the requirements to observe the AIF-induced surface projections. It required energy, as it was not observed under conditions of energy depletion (in the presence of 2-deoxyglucose and sodium azide); it required actin polymerization, as it was not observed in the presence of cytochalasin D (see Fig. 10). Brefeldin A pretreatment, however, did not inhibit the AIF response, consistent with the lack of effect of brefeldin A on the distribution of ARF6 in cells (Peters et al., 1995).

**Protrusions Induced by AIF Resemble Those Formed in Cells Expressing Q67L, the GTPase-defective ARF6 Mutant**

We next sought to determine whether the morphologic change we were observing in AIF-treated, wild-type ARF6-expressing cells was similar to the effect of expressing the GTPase-defective mutant of ARF6, Q67L, in cells alone.
If the AIF treatment acted in part to shift the wild-type ARF6 protein into the GTP form, then protrusions should be observed in cells expressing the Q67L mutant. When HeLa cells were transfected with HA-tagged Q67L, as early as 16 h, some transfected cells could be observed exhibiting membrane protrusions. By 24 h, nearly all cells transfected with Q67L elaborated projections from the cell surface in the absence of AIF treatment (Fig. 2), whereas the untransfected HeLa cells remained flattened and polygonal in shape. The distribution of the Q67L ARF6 appeared diffuse in these cells and was not enriched in the protrusive structures, as was observed with AIF treatment of cells expressing the wild-type protein (Fig. 1). The diffuse ARF6 localization in the Q67L mutant may be a consequence of prolonged, constitutive activation of ARF6 in the transfected cells. In support of this, during acute activation of wild-type ARF6 with AIF, crisp localization of ARF6 and actin within the dynamic protrusive structures is observed during the first 60 min of incubation, but with extended incubations, ARF6 and actin localization becomes more diffuse (see Fig. 1; 120 min). The significance of these diffuse localizations during chronic activation and in Q67L is not known.

The transformation of normally polygonal-shaped cells into cells forming cell surface protrusions was thus induced by the expression of Q67L alone, the ARF6 GTPase-defective mutant predicted to be in a constitutively active state. The ability of AIF treatment to induce protrusions in cells expressing wild-type ARF6, thus approaching the phenotype of Q67L-expressing cells, is consistent with the AIF acting, at least in part, to shift a portion of the wild-type protein into the active, GTP state.

**Protrusion Formation Is Specific for Wild-type ARF6 Expression**

The formation of protrusions by AIF treatment was observed only in cells that were transfected with the ARF6 plasmid and not in untransfected HeLa cells. To test whether this response was specific for the ARF6 wild-type protein, we analyzed the response to AIF of HeLa cells transfected with mutant forms of ARF6 (Fig. 3). The wild-type ARF proteins all contain an amino-terminal consensus signal for myristoylation, and this lipid modification is required for biological activity in vivo (Donaldson and Klausner, 1994). Cells transfected with an ARF6 mutant, in which the myristoylation signal was abolished by substituting an alanine residue for a glycine at position 2 (G2A), did not form protrusions, as assessed by phalloidin staining (not shown), or redistribute the ARF6-G2A protein to peripheral sites during AIF treatment (Fig. 3) nor showed any changes in actin distribution (not shown). Unfortunately, the T27N mutant of ARF6, although inactive, did not reveal a dominant, negative phenotype; in cotransfection experiments with wild-type ARF6, T27N did not inhibit the AIF-induced redistribution of wild-type ARF6 to, and formation of, protrusive structures (not shown). These experiments demonstrate that to induce membrane extensions, the ARF6 protein expressed must be myristoylated and capable of GTP binding.

The formation of protrusions in response to AIF was not observed in cells overexpressing other ARF family members. Previous in vivo and in vitro studies have demonstrated the Golgi localization and functioning of the ARF1 protein (Donaldson and Klausner, 1994). When we transfected HeLa cells with wild-type ARF1 protein, we observed ARF1 localized to the Golgi complex (Fig. 3), consistent with previous studies (Dascher and Balch, 1994; Teal et al., 1994). Treatment of these cells with AIF did not significantly alter ARF1 Golgi localization and did not induce the formation of protrusions. The inability of the peripheral sites during AIF treatment (Fig. 3) nor showed any changes in actin distribution (not shown). Unfortunately, the T27N mutant of ARF6, although inactive, did not reveal a dominant, negative phenotype; in cotransfection experiments with wild-type ARF6, T27N did not inhibit the AIF-induced redistribution of wild-type ARF6 to, and formation of, protrusive structures (not shown). These experiments demonstrate that to induce membrane extensions, the ARF6 protein expressed must be myristoylated and capable of GTP binding.

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![Figure 2. Expression of Q67L, the constitutively active mutant of ARF6, in cells induces cell protrusions. HeLa cells were transfected with plasmid encoding HA-tagged Q67L mutant of ARF6. 24 h after transfection, the cells were fixed and labeled for immunofluorescence as in Fig. 1. Cellular protrusions and surface extensions can be observed emanating from the transfected cells.](image-url)
Figure 3. AIF-induced protrusions are specific to cells expressing wild-type ARF6. HeLa cells transfected with plasmids encoding either HA epitope-tagged wild-type ARF6, ARF6/G2A, ARF6/T27N, or ARF1 were incubated in the presence (+AIF) or absence (Untreated) of AIF for 30 min, fixed, and processed for indirect immunofluorescence. AIF treatment induced protrusions only in cells transfected with wild-type ARF6.

The Racl protein was diffusely distributed in untreated cells overexpressing Racl (Fig. 4). Treatment of these cells with AIF, however, resulted in a distinctive redistribution of both Rac and actin staining to membrane folds present over the surface of the cell. This phenotype approached that of cells transfected with the GTPase-defective mutant of Rac (Q61L) where numerous membrane folds and ruffles were observed on the surface of the transfected cells, labeled by both anti-Rac antibody and phalloidin. These membrane ruffles, observed in HeLa cells, were reminiscent, in part, of the ruffling response observed in serum-starved Swiss 3T3 cells microinjected with activated Rac proteins (Ridley et al., 1992). The structure of these actin-rich plasma membrane alterations was clearly different from that seen in ARF6-transfected cells. Whereas activated Rac resulted in numerous membrane folds over the entire surface of the membrane, activated ARF6 induced folds of membrane at discrete sites around the periphery of the cells, protruding outward.

Characterization of AIF-induced Surface Protrusions

Rapid changes in cell shapes, such as the extension of the protrusions observed in the ARF6 transfected cells, usually involve the activation and recruitment of numerous signaling molecules, as well as actin-associated proteins in-
EE epitope antibody followed by fluorescein donkey anti-mouse epitope-tagged wild-type Racl or Rac1/Q61L. Cells expressing were transiently transfected with plasmids encoding either EE Figure 4. Aluminum fluoride stimulates the formation of lamellopodia in HeLa cells expressing wild-type Rac 1. HeLa cells were transiently transfected with plasmids encoding either EE epitope-tagged wild-type Rac1 or Rac1/Q61L. Cells expressing wild-type Rac1 were incubated either with (+AIF) or without (Untreated) AIF for 30 min, fixed, and stained with a mouse anti-EE epitope antibody followed by fluorescein donkey anti–mouse IgG and rhodamine-phalloidin. The Rac1 protein was diffusely distributed in untreated cells. The addition of AIF to these cells resulted in the formation of extensive membrane folds over the top of the cell, similar to those observed in cells expressing the GTPase-defective Rac1/Q61L mutant.

involved in actin disassembly and reassembly (Condeelis, 1993; Stossel, 1993; Jamney, 1994). We examined whether such proteins were recruited into the protrusions in ARF6-transfected cells. In untreated cells, the calcium-regulated, actin-severing and capping protein, gelsolin, colocalized with ARF6, particularly at the peripheral edges of transfected cells, but importantly, gelsolin distribution was not disturbed by overexpression of wild-type ARF6. After treatment with AIF, gelsolin was redistributed to the protrusions along with wild-type ARF6 (Fig. 5), with a similar time course, preceding the recruitment of actin. We also examined the distribution of proteins associated with focal adhesions, sites of contact between actin stress fibers and the plasma membrane. FAK was localized in untreated cells to focal adhesions, some of which were positive for ARF6, especially at peripheral edges of cells. The extent of this colocalization increased upon AIF treatment (Fig. 6). Cortactin, a substrate phosphorylated by FAK, was observed to be mostly diffuse in untreated cells, regardless of whether they were transfected; AIF treatment induced a marked redistribution and concentration of cortactin in the protrusive structures (Fig. 6). In untransfected cells, the distribution of gelsolin, FAK, and cortactin was not significantly altered by AIF treatment. The protrusions were also labeled heavily with antibody to phosphotyrosine residues (not shown), indicative of the activation of kinases such as FAK during AIF stimulation of the cells. Although gelsolin, FAK, and cortactin were clearly concentrated in these protrusions, other proteins such as talin and vinculin were not specifically concentrated there (not shown).

The AIF-induced Membrane Protrusions Are Sites of Enhanced Macropinocytosis and Are Devoid of Clathrin

The abundance of multilamellar folds within the membrane protrusions in ARF6-transfected cells and the dynamic nature of these structures prompted us to ask to what extent plasma membrane traffic events were required or altered during AIF treatment. Since sites of plasma membrane ruffling often exhibit enhanced fluid-phase pinocytosis (Steinman et al., 1983; Bar-Sagi and Feramisco, 1986), we examined the fluid-phase uptake of FD into cells incubated in the presence or absence of AIF. The protrusions in transfected cells were visualized by labeling the surface with rhodamine-labeled LCA (Fig. 7) after fixation.

In the absence of AIF, FD was observed in scattered punctate structures after a 10-min incubation in both transfected and untransfected cells. The FD redistributed to larger structures in the perinuclear region upon further incubation (see, e.g., Fig. 7 B). This pattern of fluid-phase uptake is typical and has been described for many cell types (van Deurs et al., 1989; Watts and Marsh, 1992).

We next assessed the uptake of FD into transfected (Fig. 7 A) and untransfected (Fig. 7 B) cells pretreated with AIF for 30 min. FD was rapidly internalized within 1 min into large, discrete structures within the membrane protrusions of transfected cells treated with AIF (Fig. 7 A). These structures were reminiscent of macropinosomes that have been described in a number of cell types (Hewlett et al., 1994; Racooan and Swanson, 1992). The number of macropinosomes increased after 10 min of loading with FD and remained mostly localized within the protrusions. Smaller, punctate spots were also scattered throughout these cells but are not visible in this exposure, which was optimized to visualize the loading into the protrusions. FD was clearly internalized into these protrusive sites and not merely entrapped within the membranous folds, since no labeling was observed in cells incubated with FD at 4°C (not shown). If cells were labeled with FD for 10 min, rinsed, and then incubated in media containing AIF but no FD for 30 min, the FD was no longer associated with the visible protrusions and had apparently been released from the cells (Fig. 6 A). The FD remaining in the cell was localized in punctate structures in the perinuclear region.

In contrast with the rapid uptake and release of dextran in cells forming protrusions, untransfected HeLa cells treated with AIF showed no detectable uptake of FD after a 1-min incubation with FD, and after 10 min, small punctate structures were observed scattered about the cell periphery (Fig. 7 B), which moved into the perinuclear region after a 30-min chase in media without dextran. Thus, although fluid uptake was the same in both transfected and untransfected cells in the absence of AIF, there was a clear change in the pattern of uptake and recycling of
membrane and fluid within the membrane protrusions in AIF-treated, ARF6-transfected cells.

Given the apparent increase in fluid internalization in cells forming protrusions, we investigated whether receptor-mediated endocytosis was altered in these cells. We examined the internalization of human transferrin in cells overexpressing ARF6 in the presence and absence of AIF, using immunofluorescence. In the absence of AIF, transferrin taken up after a 30-min incubation was observed in scattered, punctate structures with increasing concentration of these structures around the nucleus (Fig. 8). This distribution was the same for both transfected and untransfected cells and was similar to what was observed for the distribution of the transferrin receptor (not shown). In the presence of AIF, transferrin was again observed in structures around the nucleus in both transfected and untransfected cells (Fig. 8). In transfected, protrusion-forming cells, transferrin could also be observed diffusely labeling the protrusions. Similar results were obtained when cells were pretreated with AIF for 30 min before the addition of transferrin and continued incubation with AIF for an additional 30 min. Importantly, neither overexpression of ARF6 nor AIF treatment blocked transferrin uptake as monitored by immunofluorescence.

The diffuse transferrin labeling of the protrusions prompted us to ask whether internalization of transferrin via clathrin-coated pits occurred in these protrusions or elsewhere along the cell surface. To address this, we examined the distribution of clathrin-coated pits along the plasma membrane in ARF6-transfected cells by monitoring the clathrin-associated protein, AP-2. We found AP-2 distributed evenly along the plasma membrane in a punctate distribution in all untreated cells, and this distribution was not altered in cells forming protrusions with AIF treatment (Fig. 9). A similar lack of effect of AIF treatment on the distribution of caveolin was also observed (not shown). The lack of any apparent "concentration" of these plasma membrane–associated coat proteins in the overlapping membrane folds of the protrusions suggests that clathrin-coated plasma membrane and caveolin were, in fact, excluded from the protrusive regions. Interestingly, in cells expressing the constitutively active ARF6 mutant Q67L, the membranous folds observed in these cells by EM are also largely devoid of clathrin-coated pits (Peters et al., 1995).

**Inhibitors of Arachidonic Acid Metabolism Block the AIF-induced Protrusions**

We searched for reagents that would either inhibit or enhance the AIF-stimulated formation of protrusions. We saw no effect of inhibiting protein or lipid kinases or phosphatases using such reagents as staurosporine, genistein, wortmanin, and okadaic acid (not shown).

A recent study demonstrated that the growth factor–induced stimulation of stress fiber formation is signaled through Rac and then Rho via arachidonic acid metabolites, specifically leukotrienes (Peppelenbosch et al., 1995). After its release from membrane lipids by phospholipase A2 (PLA2), arachidonic acid is metabolized by two distinct pathways: the cyclooxygenase pathway leading to the production of prostaglandins, and the lipoxygenase pathway leading to the formation of leukotrienes and eicosanoids. In a previous study, Peppelenbosch et al. (1993) had demonstrated that growth factor–induced stress fiber formation was blocked in the presence of specific inhibitors of the lipoxygenase pathway but not by inhibitors of the cyclooxygenase pathways.

We investigated whether arachidonic acid metabolism was involved in the AIF-stimulated formation of surface protrusions in ARF6-overexpressing cells. For these experiments, HeLa cells transfected with ARF6 were prein-
bution and formation of protrusions. The requirement for arachidonic acid release and leukotriene production was also necessary for the continued formation of protrusions, since addition of 4BPB or NDGA to cells pretreated for 30 min with AIF reversed protrusive activity (not shown).

Discussion

The ARF family of GTP-binding proteins is believed to function as regulators of membrane traffic and organelle structure. The function of the ARF1 protein has been well characterized; it reversibly associates with membranes of the Golgi complex and is required to assemble, in a GTP-dependent manner, the cytosolic coat complex, COPI, onto Golgi membranes. The regulated coating and uncoating of Golgi membrane is necessary to allow transport through the Golgi complex and the maintenance of Golgi organellar structure (Donaldson and Klausner, 1994). In contrast with ARF1, defining specific cellular functions for the other ARF family members has yet to be accomplished.

Here we report on a novel role for the ARF6 GTPase at the cell surface that is distinct from the role of ARF1 at the Golgi complex. The ability of AIF to reversibly induce cell surface, actin-enriched protrusions in cells overexpressing the wild-type ARF6 protein, similar to protrusions observed in cells expressing the constitutively active, ARF6 mutant (Q67L), suggests that ARF6, through its GTP cycle, may play a role in determining cell surface morphology. As ARF1 regulates organelle structure at the Golgi complex, it is possible that ARF6 serves a similar function at the plasma membrane.

In this study, we show that cells overexpressing the wild-type ARF6 protein form dynamic, multilamellar, actin-rich protrusions in response to acute treatment with AIF. A number of observations suggest that the AIF treatment is acting, in part, to shift the nucleotide status of the ARF6 protein into the GTP state, enabling us to observe the ARF6 effector function. First, protrusions are formed with AIF treatment only in cells expressing wild-type ARF6 protein, capable of being activated with GTP, and not in cells expressing nonmyristoylated ARF6 (G2A) or the GTP-binding-defective mutant (T27N), and not in cells overexpressing wild-type ARF1 or ARF5 (Fig. 3). Second, the cellular protrusions exhibited by cells treated with AIF resemble protrusions observed in cells expressing the GTPase-defective mutant of ARF6, Q67L (Fig. 2). Immunelectron microscopy has confirmed that 30 min of AIF treatment of cells expressing wild-type ARF6 results in increased localization of ARF6 at the plasma membrane and the extension of numerous plasma membrane folds (Peters, P., personal communication) resembling the membrane elaborations observed, by EM, in cells expressing the constitutively active Q67L mutant (Peters et al., 1995). Finally, and remarkably, we observed that AIF treatment had a similar activating effect on cells overexpressing another GTPase implicated in actin rearrangements, the Rac1 protein. AIF treatment induced membrane ruffles in cells overexpressing Rac1, similar to those observed in cells expressing the constitutively active, Q61L. Rac mutant (Fig. 4). Thus, for both ARF6 and Rac1 in this system,
Figure 7. Macropinosomes rapidly form and recycle to the plasma membrane within protrusions of ARF6-expressing cells. Transfected (A) and untransfected (B) HeLa cells were treated with AIF for 30 min, and then 3 mg/ml FD was added for 1 min, 10 min, or 10 min followed by a 30-min chase in the absence of FD but continued presence of AIF. The cells were fixed, and the surface was labeled with rhodamine-labeled lens culinaris aglutinin (LCA).

AIF treatment appears to result in a phenotypic shift to the GTP-active form. Confirmation of this point will require determining the guanine nucleotide state of ARF6 in the cell after AIF treatment, currently under investigation.

Although the cell surface protrusions observed in AIF-treated cells transfected with the wild-type ARF6 were similar to those observed in cells expressing the constitutively active ARF6, Q67L, they were not identical. It is not surprising that the phenotypes observed in the two instances would not be the same. In one case, normal, polygonal-shaped cells expressing wild-type ARF6, which can cycle between a GTP- and GDP-bound state, are treated with AIF, which presumably results in an acute shift to the GTP-active state and the formation of surface protrusions. AIF is probably also activating other signaling pathways in the cells, some of which may enhance the protrusive activities of the transfected cells during AIF treatment. In contrast, cells expressing Q67L, the GTPase-defective mutant, are expressing an ARF6 that is locked into the GTP-active state. Expression of this irreversibly active mutant does result in the formation of protrusions early in the transfection, but other aspects of the cell morphology and the fuseness of the Q67L localization are difficult to interpret. It is for these reasons that we favor studying ARF6 function in cells expressing the wild-type protein, where the induction of protrusive activity in these cells by AIF is rapid at the onset and is reversible, enabling us to study the full GTP cycle of the ARF6 protein.

The cellular target, or targets, of AIF involved in the formation of these cellular protrusions is not known. Although AIF has been shown to directly activate trimeric G proteins when GDP is bound to the α subunit (Higashijima et al., 1991), there is no evidence that AIF can directly activate any low molecular weight GTPase in this manner (Kahn, 1991). Nevertheless, AIF treatment has been shown to protect ARF1-GTP from GTP hydrolysis.
Figure 8. The internalization of transferrin is not blocked in ARF6-expressing cells. Transfected cells were incubated at 37°C with 30 μg/ml iron-saturated human transferrin for 30 min either in the absence (Untreated) or presence (+AIF) of AIF, fixed, and processed for immunofluorescence. ARF6 was labeled with a mouse anti-HA antibody and transferrin was labeled with rabbit antiserum to human transferrin.

in vitro, in the presence of Golgi membranes (Finazzi et al., 1994). Given the many examples where actin changes are induced by G protein-coupled receptors (see Stossel, 1993) and other observations made suggesting a link between ARF and trimeric G proteins (Donaldson et al.,

Figure 9. The plasma membrane clathrin adaptors, AP-2, are not redistributed to protrusions during AIF treatment of ARF6-expressing cells. Transfected cells were untreated or treated with AIF (+AIF) for 30 min at 37°C, fixed, and processed for double-label immunofluorescence. Cells were stained with rabbit anti-ARF6 serum and a mouse antibody to α-adaptin, followed by appropriate fluorescently labeled donkey antibodies. AP-2 was distributed in a discrete punctate pattern, which did not become concentrated in protrusions after the addition of AIF (arrow).

Figure 10. Stimulation of protrusions by AIF is blocked by inhibitors of actin polymerization and arachidonic acid metabolism. Transfected HeLa cells expressing wild-type ARF6-HA were either not preincubated (AIF alone) or preincubated for 10 min with 0.2 μM cytochalasin D (Cyto D) or with 20 μM 4-BPB, NDGA, or indomethacin before incubation with AIF for an additional 30 min. Cells were fixed and processed for immunofluorescence. Protrusions were easily detectable in ARF6-expressing cells treated with AIF alone or those treated with the cyclooxygenase inhibitor, indomethacin. In contrast, treatment of cells with Cyto D, the PLA2 inhibitor, 4-BPB, or the lipoxygenase inhibitor, NDGA, completely inhibited the formation of protrusions in response to AIF stimulation.

1991; Ktistakis et al., 1992; Colombo et al., 1995), it seems reasonable to ascribe a G protein involvement in the mechanism whereby AIF causes formation of these protrusions in ARF6-expressing cells. However, fluoride ions in combination with aluminum can also affect various ATPases and phosphatases (Chabre, 1990). Regardless of the mechanism of action of AIF and the potential AIF targets, the response we are monitoring is selective, only observed in cells overexpressing the wild-type ARF6 and thus ARF6 dependent, and is fully reversible.

The AIF-induced, ARF6-dependent protrusion formation was blocked by inhibitors of arachidonic acid metabolism. The inhibition by NDGA, and not by indomethacin, suggests that the lipoxygenase arm of arachidonic acid me-
tabolism, involved in leukotriene production, may be required to couple the AIF stimulation of the cells to the formation of protrusions. Prostaglandin synthesis through the cyclooxygenase pathway does not appear to be necessary. Blockage of the pathway further upstream, by 4BPB, an inhibitor of PLA2, independently suggests the importance of this biochemical pathway for AIF-induced, ARF6-dependent protrusive structures. The significance of these observations, as well as their specific role in this response, will require further studies.

This study implicates the involvement of ARF6, a non-Rho-related GTPase, in cytoskeletal rearrangements that lead to the formation of cell surface protrusions. Previous studies of Hall and colleagues (Hall, 1994) have demonstrated that specific Rho-related GTPases regulate the formation of distinctive, actin-dependent structures. Thus, Cdc42 activation is associated with filopodia, Rac1 with lamellipodia, and RhoA with stress fiber formation.

The protrusions formed in ARF6-expressing cells are distinct from membrane ruffling observed in Rac-expressing cells. ARF6-dependent protrusions were not directly dependent upon Rac, based on the inability of the Rac GTP–binding-defective (T17N) mutant to inhibit ARF6 function (unpublished observations). However, we cannot rule out indirect effects or crosstalk between these two GTPases. Unfortunately, since the ARF6 GTP–binding-defective mutant, T27N, is not a dominant-negative mutant, we could not assess whether the Rac response was dependent upon ARF6 function. The observation that the three Rho GTPases can act either in a cascade or individually through selective stimulation (Kozma et al., 1995; Nobes and Hall, 1995) attests to the complexity of interaction of these cytoskeletal regulators. Identification of the cellular targets for cdc42, Rac, Rho, and ARF6 may clarify the mechanisms whereby they orchestrate these specific actin filament rearrangements.

The specific biochemical activities regulated by ARF6 are not known. The demonstration that ARF activates phospholipase D (PLD) when added to permeabilized cells (Cockcroft et al., 1994), to partially purified enzyme preparations (Brown et al., 1993), and, more recently, to cloned, recombinant human PLD1 (Hammond et al., 1995) has raised the possibility that ARF6 may be functioning in cells by affecting phospholipid metabolism. The stimulation of PLD activity has been observed using recombinant ARFs 1, 5, and 6 (Massenburg et al., 1994), raising the possibility that ARF6 may function in cells through its effects on PLD. The positioning of ARF6 at the plasma membrane makes it a prime candidate as a potential activator of PLD in response to agonist and growth factor stimulation. Further investigations are needed to address this possibility.

During AIF-induced protrusion formation, we observed a stimulation of fluid uptake by macropinocytosis. Although the association of stimulated macropinocytosis with active membrane ruffling is well documented (Bar-Sagi and Feramisco, 1986; Racoosin and Swanson, 1992; Swanson and Watts, 1995), its physiological significance is unclear. In our studies, fluid internalized into macropinosomes was not further transported into the cell, but was apparently released back out into the media. The high concentration of F-actin within the protrusions may be a barrier to the further transport of the macropinosomes into the cell. In our system, this membrane pathway of stimulated fluid internalization and release may be involved in coordinating the observed changes in plasma membrane morphology with the dynamic assembly/disassembly of actin to form these protrusions. How this regulated pathway of macropinocytosis relates to that of receptor-mediated endocytosis is a topic of current research (Hansen et al., 1993; Hewlett et al., 1994) and discussions (Sandvig and van Deurs, 1994; Lamaze and Schmid, 1995).

In contrast with the stimulation of macropinocytosis observed in cells forming protrusions, we did not detect an alteration in the uptake of transferrin either by overexpression of wild-type ARF6 alone or during AIF treatment using immunofluorescence. On the other hand, D’Souza-Schorey et al. (1995) reported, using a quantitative assay, an inhibition of transferrin uptake associated with overexpression of either wild-type ARF6 alone or the Q67L mutant of ARF6 in CHO cells. A direct comparison between the two studies is difficult given the different cell types, expression systems, and assays used. It is possible that the increased membrane cycling due to macropinocytosis, as reported here, under certain circumstances, could have effects on the endosomal-transferrin cycle, but we could not detect such changes. The observation that endosomally associated ARF6 only partially overlaps with the transferrin receptor (Peters et al., 1995) suggests that the membranes through which ARF6 cycles may partially overlap between these two cycling membrane systems. Further studies will be needed to resolve these differences.

In this study, we describe a novel function for an ARF GTP-binding protein, i.e., initiating rearrangements of the actin cytoskeleton and plasma membrane to generate cell surface protrusions. Thus, ARF6 and the Rho-related GTPases may specifically regulate distinctive actin-based cell surface structures. The differential regulation of the activity of these GTPases, in conjunction with the assembly of numerous structural proteins, will ultimately determine cellular morphology. Defining the pathways whereby each GTPase functions, the regulation of actin assembly, and the role of membrane traffic in the modeling of the cell surface will provide a major challenge to cell biologists.

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