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Regulation of epithelial junctions by proteins of the ADP-ribosylation factor family

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1. ABSTRACT

ADP-ribosylation factor (ARF) proteins play a pivotal role in the regulation of membrane traffic and the organization of the cytoskeleton that are crucial to fundamental cellular processes, such as intracellular sorting/trafficking of newly synthesized proteins and endocytosis/exocytosis. In epithelial junctions, the ARF proteins are intimately associated with the dynamics of transmembrane proteins, such as E-cadherin and beta-1 integrin, and the adaptor proteins such as paxillin. In addition, ARF proteins play a key regulatory role in the remodeling of actin cytoskeleton necessary for the formation of membrane ruffles and protrusions in association with phospholipase D and members of the Rho GTPase family. These activities of ARF proteins influence not only the formation, stability and functional integrity of epithelial junctions but also the cell motility as well. In this review, I have attempted to provide a compendium of evidence that has contributed to our evolving understanding of these ARF proteins as well as their regulators (guanine nucleotide exchange factors and GTPase-activating proteins) in the regulation of epithelial junctions. In addition, I also have also highlighted potential mechanisms as to how these intricate regulatory pathways are regulated both spatially and temporally.

2. INTRODUCTION

In this review, I will try to recapitulate the emerging evidence that dictates a pivotal role for ARF proteins in the formation and function of epithelial junctions. In epithelial tissue, cells are tightly bound together and are organized into sheets called epithelia. In epithelia, the cells are attached to each other by cell-cell adhesions, which bear most of the mechanical stresses. Specialized cell junctions occur at points of cell-cell and cell-matrix contacts in all tissues, and they are particularly ubiquitous in epithelia. Based on the nature of interface, the cell-cell contacts are classified into tight junctions (TJs), adherens junctions (AJs), gap junctions or desmosomes, whereas cell-matrix adhesions are classified as hemidesmosomes or focal adhesions/contacts (FAs). Small GTPases of Ras superfamily play a pivotal role in the epithelial biogenesis. They mediate an increasingly complex interplay between cell-cell adhesion molecules and fundamental cellular processes such as cytoskeletal activity, vesicular trafficking, cellular polarization and stabilization. In addition, they are also involved in the pathologic cellular processes of cellular transformation such as epithelial-mesenchymal transformation (1, 2).

Here, I will focus on the role of ARF proteins, a family of small GTPases, in the biogenesis and function of

Table 1. Human ARF GEFs

Group	Gene Symbol	Names	Substrates
GBF/BIG	GBF1	GBF1	ARF1, 3, 5
	ARFGEF1	BIG1, p200	ARF1, 3
	ARFGEF2	BIG2	ARF1, 3
PSCD/Cytohesins	PSCD1	Cytohesin-1	ARF1, 6 (?)
	PSCD2	Cytohesin-2, ARNO	ARF1, 3, 6
	PSCD3	Cytohesin-3, GRP1	ARF1, 6
	PSCD4	Cytohesin-4	ARF1, 5
PSD/EFA6s	PSD	EFA6A	ARF6
	PSD2	EFA6C	ARF6
	PSD3	EFA6D	ARF6
	PSD4	EFA6B	ARF6
IQSEC/BRAGs	IQSEC1	BRAG2, GEP100	ARF6
	IQSEC2	BRAG1	ND
	IQSEC3	BRAG3	ND
Fbox	Fbox8	Fbox8	ND

ND, not determined, Modified from References 11 and 16

epithelial junctions. I will discuss how ARF proteins regulate the molecular composition at the epithelial junctions and how this regulation by ARF proteins influences the function of epithelial junctions and cellular activity. Obviously this is an extremely complex field and many aspects of these intricate regulatory pathways remain to be elucidated. Accordingly, I will strive to highlight key issues that challenge the field, in the hope that such discussions might stimulate and foster further research and collaborations across different disciplines.

3. ADP-RIBOSYLATION FACTOR FAMILY

3. 1. ARFs

ARF proteins are a family belonging to the Ras superfamily of small GTPases (3). ADP-ribosylation factors (ARFs) were initially purified as cofactors for cholera-toxin catalyzed ADP-ribosylation of the alpha-subunit of heterotrimeric G proteins, Gs, and then identified as GTP binding proteins *in vitro* assays (4, 5). However, now it is well established that ARF family is involved in many intracellular processes including vesicular biogenesis and intracellular trafficking (reviewed in 6-10).

ARF proteins are soluble proteins that generally associate with membranes via N-terminal myristoylation and are ubiquitous in all eukaryotes from yeast to humans. Currently, ARF family is composed of six mammalian ARFs and many other ARF-like proteins (reviewed in 11, 12). A total of 29 members have been identified in humans. The six principal mammalian ARF members are divided into three classes based on their amino-acid sequence similarity. Class I ARF members (ARF1, ARF2 and ARF3) share among themselves more than 94% amino-acid sequence homology. They localize to the Golgi apparatus and are involved in the trafficking of endoplasmic reticulum - Golgi and endosomal systems. Class II ARF members, ARF4 and ARF5, are 90% identical to each other and show approximately 80% identical with that of class I ARFs. They also localize to the Golgi apparatus as class I ARFs but are less abundant than class I ARFs. In contrast to class I ARFs, the function of class II ARFs remains poorly understood. Class III has only a solitary member, ARF6 that shares 65 – 70% amino acid homology with other ARFs and localizes at the cell periphery.

3.2. Regulators of ARFs: guanine nucleotide exchange factors and GTPase-activating proteins

Similar to other Ras-related GTP-binding proteins, the ARF proteins cycle between GTP-bound active state and GDP-bound inactive state. ARFs bind to GTP tightly but have a very low intrinsic activity of GTP hydrolysis (5). Their GTPase cycle is regulated by guanine nucleotide exchange factors (GEFs) that exchange GDP for GTP on ARFs lead to their activation. Conversely, GTPase-activating proteins (GAPs) that hydrolyze bound GTP to GDP deactivate these ARFs.

All ARF GEFs share a 200-amino acid, Sec 7 domain module, that catalyzes the exchange of GDP for GTP on ARFs, thus, being referred to as the Sec7 family. The fungal fatty acid metabolite brefeldin A (BFA), which disassembles the Golgi complex and blocks protein secretion, directly inhibits some of ARF GEFs, but not all. BFA binds to the ARF-GDP-ARF GEF complex, and prevents the conformational changes that are required for GEFs to release the bound GDP, resulting in failure to activate ARFs (13-15). So far, there are fifteen members of human ARF GEFs that have been identified and these are classified into five groups, GBF/BIG, PSCD/Cytohesins, PSD/EFA6s, IQSEC/BRAGs and Fbox, based on their overall structure and domain organization (Table 1)(16).

All known ARF GAPs contain a well-conserved ARF GAP domain, that is necessary for GAP activity. In mammals, at present, 24 proteins with ARF GAP domains have been identified and these 24 members are classified into two types, ArfGAP and AZAP, based on their overall structure and each type is further subdivided according to domain organization (Table 2)(17). Originally ARF GAPs were identified as negative-regulators of ARFs and they have been implicated in membrane trafficking of vesicle coats by inactivating ARF activity (18, 19). These ARF GAPs are structurally complex and contain multiple domains/motifs in addition to their ARF GAP domain that is common to all the members. Through these complex domains, ARF GAPs interact with a variety of proteins and have been shown to be involved in many different intracellular processes and functions including those of membrane trafficking, regulation of actin cytoskeleton and signaling both dependently and independently of ARFs.

Table 2. Human ARF GAPs

Type	Group	Gene symbol	Names	Substrates
ArfGAP	ArfGAP	ARFGAP1	ArfGAP1	ARF1
		ARFGAP3	ArfGAP3, ARFGAP1	ARF1
	SMAP	SMAP1	SMAP1	ARF6
		SMAP1L	SMAP2	ARF1
	GIT	GIT1	GIT1, p95-APP1, Cat1	ARF1, 6
		GIT2	GIT2, p95-APP2, Cat2	ARF1, 6
AZAP	ASAP	DDEF1	ASAP1, AMAP1, PAG2	ARF1, 5
		DDEF2	ASAP2, AMAP2, PAG3	ARF1, 5, 6
		DDEFL1	ASAP3	ARF5
	ACAP	CENTB1	ACAP1	ARF6
		CENTB2	ACAP2	ARF6
		CENTB5	ACAP3	ND
	ARAP	CENTD1	ARAP2	ARF6
		CENTD2	ARAP1	ARF1, 5
		CENTD3	ARAP3	ARF5, 6
	AGAP	CENTG1	AGAP2, PIKE	ARF1, 5
		CENTG2	AGAP1	ARF1, 5
		CENTG3	AGAP3	ND

ND, not determined, Modified from References 11, 17 and 51

The presence of a large number of ARF GEFs and ARF GAPs that are capable of modulating the activities of relatively few ARFs implies a complex and intricate regulatory mechanism operational in various subcellular compartments to coordinately regulate ARFs temporally or spatially in response to various stimuli or upstream events.

Considering the fact that a single molecule may be referred to by several different names in the published literature, the nomenclature of ARF GEFs and ARF GAPs in this review represents gene symbol/alternate name given in published reports for each ARF GEF or ARF GAP.

3. 3. Functional properties of ARFs

Among the six ARF members, ARF1 and ARF6 are the most extensively studied. ARF1 localizes to the Golgi complex and has been implicated in the endoplasmic reticulum to Golgi transport, organization and function of the Golgi, transport from the trans-Golgi network, transport in the endocytic pathway and regulation of enzymes that modify membrane phospholipids (6, 7, 20-26).

Unlike ARF1, ARF6 has no effect on Golgi membrane dynamics. ARF6 localizes at the cell periphery and is implicated in the regulation of plasma membrane–endosomal trafficking and structural organization of the peripheral plasma membrane, such as endocytosis/recycling of membrane proteins (27-30), exocytosis/secretion of proteins (31-33), phagocytosis (34-36), cell migration (37-40), cell spreading / scattering (41, 42), Rac-induced membrane ruffling (40, 43, 44), actin cytoskeleton remodeling (45, 46), neurite outgrowth (47, 48) cytokinesis and in the activation of enzymes that modify membrane phospholipids (49, 50).

There are several excellent and comprehensive reviews that discuss the cellular functions of ARFs, ARF GEFs and ARF GAPs for those readers who are interested in an in-depth view (6-9, 16, 17, 51).

4. TIGHT JUNCTIONS

Tight junctions (TJs) are a type of occluding junctions found in vertebrates and these TJs seal neighboring cells together in an epithelial sheet to prevent even small molecules and water from passing through paracellular pathways. They serve as barriers between the apical and basolateral membranes, which is one of the basic functions of epithelia. TJs also prevent the diffusion of membrane proteins and lipids which are important in the maintenance of cell polarity and directional transport between apical and basolateral domains. TJs are composed of several proteins including transmembrane proteins such as claudins and occludin, the scaffold proteins such as cingulin and ZO proteins, with which transmembrane proteins interact (52).

Polarized Madin-Darby canine kidney (MDCK) cells that overexpress PSD/EFA6A, a GEF specific for ARF6, show accelerated kinetics of the assembly or a delay in the kinetics of disassembly of TJs (53). This effect requires the Sec7 domains and the C-terminal domains of PSD/EFA6A that are essential for the GDP-GTP exchange activity and membrane localization, respectively, suggesting that this effect of PSD/EFA6A on TJs requires ARF6 activation. In addition, selective retention of occludin at the cell surface along with strong apical actin and ZO-1 accumulation were observed in these cells overexpressing PSD/EFA6A, indicating that PSD/EFA6A enhances the stabilization of protein complexes including actin cytoskeleton at the TJs. The precise molecular mechanism responsible for this effect remains unclear, however, PSD/EFA6 is involved in the regulation of endocytic vesicles as well as actin remodeling (54-56) and it has been shown that PSD/EFA6 promotes the redistribution of membrane receptors to the cell surface by regulating ARF6 and Rac1 (55). This retention of occludin imparts stability to the TJs. Indeed, the TJs in cells overexpressing PSD/EFA6A were more stable than in controls cells (53). Cell polarity was retained in MDCK cells overexpressing PSD/EFA6A or a GTPase-defective ARF6 mutant,

ARF6(Q67L) (37, 53). In further support of ARF6 and PSD/EFA6A involvement in TJs, the overexpression of wild-type ARF6 in MDCK cells led to its localization to the apical surface of these cells (28) while the overexpression of PSD/EFA6A resulted in the distribution of this protein at TJs along with ZO-1 protein (53).

5. ADHERENS JUNCTIONS

Adherens junctions (AJs) are a type of anchoring junctions that are most abundant in tissues subjected to severe mechanical stress. They also play a key role in the formation and maintenance of TJs and desmosomes. AJs consist of two basic adhesive units: classical cadherin-catenin complex and nectin-afadin complex (57). In the classical cadherin-catenin complex, the intracellular anchor proteins, such as catenins, vinculin, and alpha-actinin, form a distinct plaque on the cytoplasmic face of the plasma membranes and connect the junctional complexes to actin cytoskeleton. Transmembrane adhesion proteins of the cadherin-catenin complex belong to the cadherin family. In the nectin-afadin complex, nectin, a transmembrane IgG-like adhesion protein, forms a structural adhesive unit with afadin, which is an actin-binding protein. However, at the present, there is no evidence to implicate ARFs in the regulation of nectin-afadin complexes.

5. 1. E-cadherin

E-cadherin is the predominant cadherin found in the epithelial cadherin-catenin complexes. The newly synthesized E-cadherin binds to beta-catenin and gets transported to lateral membranes of the epithelial cells to be assembled into AJs (58). E-cadherin on the epithelial cell surface undergoes constitutive internalization to enter early endosomal compartments (59- 62) and then gets recycled back to surface plasma membranes. In addition to being constitutively active in polarized cells, the disruption of AJs and internalization of E-cadherin occur during cell scattering induced by growth factors (37). A similar loss of E-cadherin function and disassembly of AJs are seen in epithelial tumor invasion and metastasis (37, 57, 63).

The regulation of E-cadherin by ARF6 has been extensively studied and well documented. Activation of ARF6 is required for hepatocyte growth factor (HGF)-induced scattering activity of MDCK cells (37, 42). Activation of ARF6 in MDCK cells promotes clathrin-dependent internalization of E-cadherin, resulting in the disassembly of AJs, (37). Similar evidence has been found in MCF-7 cells that overexpress the GTPase-defective ARF6 mutant, ARF6(Q67L) (61). The molecular mechanism underlying the ARF6 involvement in the AJ disassembly operates via recruitment of Nm23-H1. Nm23-H1 is a nucleoside disphosphate kinase that facilitates dynamin-mediated internalization of E-cadherin during AJ disassembly. Nm23-H1 also decreases Rac1-GTP levels at the cell junctions (59). Activation of Rac1 has been implicated in the accumulation of actin at the AJs (64-66). Thus, the downregulation of Rac1 by Nm23-H1 probably leads to a decrease in actin fibers at the AJs and also facilitates the disassembly of these AJs. Consistent with this notion, a demonstrable reduction in actin staining has been reported during ARF6-mediated disassembly of AJs (37).

A GEF for ARF6 (IQSEC1/BRAG2/ GEP100), was also shown to be involved in the regulation of E-cadherin (67). Ablation of IQSEC1/BRAG2/GEP100 expression by siRNA treatment in hepatocellular carcinoma HepG2 cells results in an increase of E-cadherin protein levels and also alters its intracellular distribution after HGF treatment (68). In control cells, E-cadherin was internalized and diffusely distributed in the cytoplasm upon HGF treatment, while in where IQSEC1/BRAG2/GEP100 knockdown cells, E-cadherin remained at the cell periphery, suggesting that IQSEC1/BRAG2/GEP100 is likely to be involved in the HGF-induced internalization of E-cadherin. Recently, IQSEC1/BRAG2/GEP100 has also been implicated in the cell invasiveness of breast cancer cells (69). In this study, co-overexpression of ARF6 and IQSEC1/BRAG2/GEP100, but not its mutant lacking the Sec7 domain, in breast cancer cells resulted in the impairment of E-cadherin-mediated cell-cell adhesion after treatment with epidermal growth factor (EGF). In addition, co-overexpression of ARF6 and IQSEC1/BRAG2/GEP100 induced invasive activity upon EGF stimulation. However, PSCD2/Cytohesin-2/ARNO, one of the other GEFs that regulate ARF6, did not result in any invasive activity. Moreover, overexpression of PSD/EFA6A also failed to demonstrate any effect on intracellular distribution of E-cadherin in MDCK cells (53). Taken together, these observations suggest that ARF6 in conjunction with IQSEC1/BRAG2/GEP100 play an important role in the regulation of E-cadherin-mediated cellular functions. In MDCK cells that overexpressed a dominant negative ARFGEF2/BIG2 mutant, ARFGEF2/BIG2(E738K), the E-cadherin-beta-catenin complexes at the AJs were disrupted and these disrupted E-cadherin-beta-catenin complexes co-localized with the Golgi maker GM130 in the perinuclear cytoplasm, suggesting that E-cadherin and beta-catenin were arrested in the Golgi in such cells (70). ARFGEF2/BIG2 predominantly activates class I ARFs (ARF1 and ARF3)(71). Thus, ARFGEF2/BIG2 and probably class I ARFs are involved in the transport of E-cadherin and beta-catenin from the Golgi apparatus to the cell surface.

5.2. Alpha-catenin

In the cadherin-catenin complexes, alpha-catenin interacts with E-cadherin via beta-catenin and also binds to actin. Alpha-catenin was long considered to be a linker between the AJs and actin fibers but more recent evidence suggests that alpha-catenin is likely to serve as a molecular switch that coordinates the functions of AJs in the cell–cell interactions as well as actin cytoskeleton in the cell motility (72-74).

Alpha-catenin was identified as an IQSEC1/BRAG2/GEP100-binding protein in yeast two

hybrid screens (68). This physical interaction between alpha-catenin and IQSEC1/BRAG2/GEP100 was confirmed by coimmunoprecipitation studies, but not by immunofluorescent assays. The ablation of IQSEC1/BRAG2/GEP100 expression by siRNA resulted in increased levels of alpha-catenin in the cells (68). In the presence of alpha-catenin, GTP binding activity of ARF6 which is IQSEC1/BRAG2/GEP100 dependent, increases modestly in *in vitro* studies (68). These findings thus imply that alpha-catenin, ARF6 and IQSEC1/BRAG2/GEP100 form a functional ternary regulatory complex but direct evidence for their intracellular physical association remains elusive.

5. 3. Vinculin and alpha-Actinin

Both vinculin and alpha-actinin are cytoskeletal proteins that associate with AJs as well as focal adhesions (FAs), and serve as linker proteins. Vinculin has binding sites for several molecules, including talin, alpha-actinin, alpha-catenin, paxillin and actin. The activities of vincluin and alpha-actinin are regulated by phosphatidylinositol (4, 5)-bisphosphate (PI(4,5)P2) and phospholipase D2 (PLD2) (75-81).

Only limited information exists in literature in support of ARF involvement in the regulation of vinculin and alpha-actinin functions. It has been shown that the intracellular distribution of vinculin is not altered by ARF1 or DDEF1/ASAP1/PAG2, one of GAPs (82, 83). In yeast two-hybrid screens with an adult mouse brain cDNA library, alpha-actinin was identified as a possible interacting protein with PSD/EFA6A, and partly colocalized with PSD/EFA6A in mouse brain tissues as well as cultured hippocampal neurons as determined by immunofluorescent studies (84). Another actin binding protein, alpha-catenin, as mentioned above, has also been identified as an interacting protein of IQSEC1/BRAG2/GEP100 (68). Both PSD/EFA6A and IQSEC1/BRAG2/GEP100 are ARF GEFs specific for ARF6 which has been demonstrated to be involved in actin remodeling. The physiological significance of the binding of these ARF6 specific GEFs to actin-binding proteins remains to be established. In *in vitro* binding analyses, ARF1 disrupted the interaction between PLD2 and alpha-actinin in a concentration-dependent manner and ablated the down-regulation of PLD2 activity by alpha-actinin (81). PLD2, which is predominantly associated with the plasma membranes, has been implicated in the cytoskeletal dynamics (85). Phosphatidic acids, a PLD2 metabolite, can enhance PI(4,5)P2 levels by activation of type I phosphatidylinositol phosphate 5 kinase (PIP5K). PI(4,5)P2, on the other hand, regulates both actin-binding activity of alpha-actinin as well as cytoskeletal dynamics. Therefore, regulation of PLD2 activity by alpha-actinin is likely to affect both actin skeletal dynamics as well as alpha-actinin activity itself. The regulatory loop of alpha-actinin and PLD2 is also likely to be impacted by ARF1 (81).

6. DESMOSOMES

Desmosomes are a type of anchoring junctions particularly important for maintaining the integrity of tissues that endure physical stress. These junctions have a dense cytoplasmic plaque composed of intercellular anchor protein complexes, such as plakoglobin, desmoplakin and plakophilins, that are responsible for connecting the cytoskeleton to transmembrane adhesion proteins, for example desmogleins and desmocollins (86).

ARF6 regulates plasma membrane–endosomal trafficking of various proteins located in the plasma membranes, such as major histocompatibility complex class I (MHC I) and various receptors (29, 30, 87-89). In HeLa cells overexpressing GTPase-defective ARF6 mutant, ARF6(Q67L), plakoglobin and MHC I were found in the actin-rich vacuolar compartment induced by ARF6(Q67L) (90), suggesting the possibility that the plasma membrane–endosomal dynamics of plakoglobin is regulated by ARF6.

7. FOCAL ADHESIONS

Focal adhesions (FAs) are a type of anchoring junctions found in the cell-extracellular matrix (ECM) interface. FAs serve as mechanical linkages to the ECM, as well as biochemical signaling centers. Therefore, numerous proteins including those of structural proteins, enzymes and adaptor proteins are concentrated and directed to the FAs. Transmembrane adhesion proteins that structurally support FAs belong to the integrin family. The intracellular domains of integrins bind indirectly to bundles of actin filaments via intracellular anchor proteins talin, alpha-actinin, filamin and vinculin. Many other intracellular proteins that are associated with signaling, such as focal adhesion kinase (FAK) and PKC, also bind to and associate with this integrin-adaptor protein-cytoskeleton complexes, thus forming the basis of FAs. The dynamic assembly and disassembly of focal adhesions play a central role in the cell migration. During cell migration, both the composition and the morphology of these FAs undergo striking changes (91).

7.1. Integrins

Integrins are heterodimeric transmembrane glycoproteins comprising of diverse alpha- and beta subunits (92). Twenty-four different alpha subunits are known to exist that can link in many different combinations with nine different beta subunits. In epithelial cells, the predominant subunit is beta1-integrin.

ARF6 controls recycling of beta1-integin from internal compartments to plasma membrane (93). The expression of a dominant-negative mutant ARF6(T27N) had no effect on the internalization of beta1-integin to recycling endosomes but increased internal beta1-integin, that significantly colocalized with ARF6. Another ARF6 mutant ARF6(Q37E/S38I), which selectively inhibits actin arrangement but not ARF6-mediated transport, also had the same inhibitory effects as ARF6(T27N) on beta1-integin recycling. A dominant-negative Rab11(S25N) also abrogated the recycling of beta1-integin, indicating that the

recycling of beta1-integin to plasma membranes is regulated by both ARF6 and Rab11 as well as requires actin cytoskeleton arrangement in an ARF6 dependent manner. Consistent with this observation, internal beta1-integin was found in the PIP2-positive vacuolar compartment induced by ARF6(Q67L) (90) and in cells with knocked down ARF6, beta1-integrin levels at the cell surface was reduced (94). Beta1-integin levels at the cell surface correlate with its function. Indeed, the cells with knocked down ARF6 spread less efficiently on fibronectin-coated substrates. CENTB1/ACAP1, that is a GAP for ARF6 as well as an effector in cargo sorting (95-97), is also shown to plays an important role in beta1-integin recycling (98). Both overexpression and abrogation of CENTB1/ACAP1 by siRNA treatment inhibited the recycling of beta1-integin, and beta1-integin was colocalized with CENTB1/ACAP1 and was accumulated in the recycling endosomes. CENTB1/ACAP1 is involved in the formation of clathrin coat complex that is regulated by ARF6 and also the cargo sorting of beta1-integirn that requires the phosphorylation of CENTB1/ACAP1 by Akt. Both are critical for beta1-integrin recycling. In contrast, CENTB2/ACAP2 did not have any inhibitory effects on beta1-integin recycling (98). There is another evidence that IQSEC1/BRAG2/GEP100 regulates beta1-integin endocytosis as well (94). The abrogation of IQSEC1/BRAG2/GEP100 expression by siRNA treatment resulted in an increase of beta1-integin at the cell surface along with more efficient and rapid spread of these cells on fibronectin-coated surfaces than control cells, suggesting the possibility that ARF6 is likely to be involved in the internalization of beta1-integin, as well as its recycling processes.

Integrin proteins are glycosylated and glycosylation is important in their cell adhesiveness and motility functions (99). Glycosylation is catalyzed by enzymes located in the Golgi appartus. In HeLa cells with knocked down ARFGEF1/BIG1, but not ARFGEP2/BIG2, aberrantly N-glycosylated beta1-integrin accumulates on the cell surface and these cells display an impairment in cell spreading and adhesion to extracellular matrix as well. Thus, it appears that class I ARFs and ARFGEF1/BIG1 are required for proper N-glycosylation of beta1-integin, affecting the functional activities of beta1-integin (100).

7.2. Filamin

Filamin A is a 280-kDa phosphoprotein that consists of two units, and cross-links actin filaments. Filamin A is widely expressed in many types of cells and regulates the reorganization of actin cytoskeleton by interacting with integrins, transmembrane receptor complexes and second messengers.

Colocalization of ARFGEP2/GIB2, a GEF for class I ARFs, and Filamin A has been demonstrated in human neural stem cells. The overexpression of a dominant negative ARFGEF2/BIG2(E738K) in neuroblastoma cells results in partial blockade of Filamin A transport from the Golgi apparatus to cell membranes (101), suggesting that the trafficking of Filamin A from the Golgi to cell membrane is, in part, regulated by class I ARFs in neural cells. The trafficking of Filamin A in epithelial cells remains unclear.

7. 3. Other proteins associated with focal adhesions

Paxillin is a signal transduction adaptor protein that localizes to FAs. Paxillin contains a number of motifs and interacts with many other proteins (102, 103). Paxillin binding proteins range from structural proteins, such as vinculin and actopaxin that bind actin directly, to regulatory proteins such as ARF GAP, p21-GTPase-activated kinase (PAK), PAK-Interactive exchange factor (PIX, GTP exchange factor for Rac1 and Cdc42). FAK is a protein tyrosine kinase that localizes to FAs, and plays a key role in the assembly and disassembly of FAs (91). FAK phosphorylates cortactin, and promotes polymerization and rearrangement of the actin cytoskeleton, which is important for lamellipodia and invadopodia formation as well as cell migration and endocytosis.

ARF1 has been shown to be involved in the recruitment of paxillin to FAs and the formation of FAs (82, 104). In COS7 cells, ARF6 activated by aluminum fluoride (AIF), changes subcellular distribution of paxillin leading to its colocalization with ARF6 at several membrane protrusions (105).

Furthermore, there is substantial evidence to show that ARF GAPs interact with several proteins associated the FAs, such as paxillin and FAK, and influence the dynamics and/or function of FAs. DDEF2/ASAP2/PAG3 directly binds to paxillin, and the overexpression of DDEF2/ASAP2/PAG3, but not its mutant lacking GAP activity, inhibits paxillin recruitment to FAs as well as cell migratory activity (105). The ARF GAP activity of CENTD1/ARAP2 or DDEF1/ASAP1/PAG2 has also been shown to influence the formation of FAs and cell spreading/migration activity (83, 106-108). In addition, DDEF1/ASAP1/PAG2 binds to FAK, tyrosin kinase Src or cortactin (83, 109, 110). Endogenous GIT1/p95-APP1/Cat1 colocalizes with paxillin at the cell periphery. The activation of GIT1/p95-APP1/Cat1 by its association with PIX, enhances its binding to paxillin and FAK, and leads to simultaneous disassembly of focal complexes and stimulation of cell motility (111). Moreover, GIT2/p95-APP2/Cat2 also localizes to FAs and binds to both paxillin and PIX as well. GIT2/p95-APP2/Cat2 mediates the association of paxillin and PAK-Nck-PIX complexes essential for PAK-dependent cytoskeleton reorganization such as lamellipodia formation (112). Many studies have also shown that GITs are involved in the formation and function of FAs (113, 114). Nonetheless, accumulating evidence has shown that ARFs and ARF GAPs play an important role in the dynamics of

paxillin and FAK associated with FAs, resulting in the control of cell motility. However the precise regulatory mechanisms remain to be elucidated. Reader is referred to several excellent and comprehensive reviews that discuss the paxillin binding proteins including ARF GAPs in greater detail for a more comprehensive account (17, 51, 115, 116).

8. CYTOSKELETON

Cell-cell adhesions bear bulk of the mechanical stress in epithelial tissues. Intercellular junctions are generally associated with cytoskeletal molecules, such as actin and intermediate filaments that span across the cytoplasm of epithelial cells. This strengthens intercellular adherence and plays a pivotal role in the junction stability. As discussed above, some membrane proteins that are integral to cellular junctions, for example, E-cadherin and beta1-integrin, have been shown to shuttle between internalization to endosomal vesicles and recycling back to the plasma membrane, constitutively and/or in response to growth factor stimulation. These cellular processes require assembly/disassembly of cellular junctional complexes, and are thought be accompanied by rearrangement/remodeling of cytoskeletal proteins.

8.1. Actin

Actin filaments are found in the AJs, TJs and FAs. Individual subunits of actin are known as globular actin (G-actin). G-actin subunits assemble into long filamentous polymers called F-actin. The members of the Rho family of GTPases, including Rac1, RhoA and Cdc42, are well known for their regulation of actin cytoskeleton organization (117, 118). It has been shown that overexpression of a dominant active mutant of Rac1 promotes the accumulation of polymerized actin at cell-cell contacts and increases E-cadherin-mediated adhesion (119).

Several studies have shown that ARF1 and ARF6 are involved in actin dynamics. ARF1 regulates the recruitment of actin to the Golgi (24, 120-123). By contrast, ARF6 is implicated in the actin remodeling at cell periphery. ARF6-regulated actin remodeling is thought to be necessary for the formation of membrane ruffles (45, 46), neurite outgrowth (47, 48), cell spreading (37), cell migration (38, 105), and phagocytosis (34, 35). The effects of ARF6 on the actin cytoskeleton are likely to be mediated through the coordination and regulation of RhoA, Rac1 GTPase activity. Indeed, ARF6 activation promotes the downregulation of RhoA, resulting in reduced stress fiber formation (44) as well as the downregulation of Rac1-GTP levels, resulting in cell scattering (42). In addition to the regulation of Rac1 and/or RhoA activity, ARF6 has also been shown to promote membrane ruffling by directly interaction with POR1, a Rac1 interacting protein, independent from Rac1 activation (45). Furthermore, ARF6 modulates the actin cytoskeleton through its effect on lipid metabolism. ARF6 activates PLD and PIP5K (49, 50), and the activation of these enzymes can directly or indirectly lead to the accumulation of PI(4,5)P2, which has been shown to regulate actin capping as well as the activities of several actin-binding proteins (124, 125). Indeed, ARF6-induced PLD activity in concert with Rac1 activation promotes cell migration (38). It has been shown that ARF6 and PSD/EFA6 influence actin remodeling at the TJs (37, 40, 53). Both ARF6 activated by aluminum fluoride (AIF) and GTPase-deficient mutant ARF6(Q67L), but not ARF1, induce active membrane ruffles and protrusions in HeLa and COS cells. Upon AIF treatment, ARF6 also promotes redistribution of FAK and cortactin at the cell periphery where the formation of membrane ruffles and protrusions occur (46). The reduced expression of CENTD1/ARAP2, a GAP that regulates ARF6, by siRNA treatment results in loss of focal adhesions and actin stress fibers (106). These observations compellingly suggest that ARF6 enhances the actin remodeling at focal adhesions and are likely to be involved in the focal adhesion turnover and function.

8.2. Intermediate filament

In epithelia, keratin intermediate filaments form desmosomes or hemidesmosomes. In mesenchymal cellular stages, epithelial cells lose their epithelial characteristics including those of AJs and cell polarity, and acquire new mesenchymal cell phenotypes, such as the expression of vimentin. Vimentin is the most widely distributed of all intermediate filament proteins, and also found in cells derived from mesoderm, such as fibroblasts, leukocytes and endothelial cells (126). The expression of vimentin is thought to be important in cell migration.

It has been shown that BFA treatment induces changes in the organization of vimentin filaments, either through retraction to the perinuclear region or through the formation of elongated process-like formations. This effect is accompanied by the release of AP1 and AP3 adaptor complexes from membranes and the binding of these adaptor proteins to vimentin. A dominant-negative mutant of ARF1, ARF(T31N), but not wild-type ARF1 or a GTPase-deficient mutant ARF1(Q71L), showed the same effect on vimentin filaments, suggesting that ARF1 is likely to participates in the alteration of vimentin cytoskeleton on the Golgi (127).

9. CONCLUDING REMARKS

Since the original discovery of ARFs being critical cofactors for cholera toxin mediated ADP ribosylation of heterotrimeric G proteins, much has been learned about these important proteins. From a cell biology perspective, a clearer picture has emerged as to how these proteins play a pivotal role in the regulation of vesicle formation and transport. With their ability to regulate lipid modifying enzymes, these proteins can influence the very composition of phospholipid membranes and the cytoskeletal organization. We have made great strides in understanding how these ARF proteins participate and regulate many

Table 3. Involvement of ARFs in cellular junctions

Junction/protein	ARFs	Regulators	Function/Remarks	Reference
TJ	ARF6	PSD	TJ stabilization	53
			retention of occludin	
			stability of actin ring	
E-cadherin	ARF6		AJ disassembly / turnover	37, 42, 59, 61
			E-cadherin internalization	
			membrane ruffling	
			cell migration, scattering	
	ARF6	IQSEC1	E-cadherin internalization	68
			binding to alpha-catenin	
			actin remodeling	
	ARF6	IQSEC1	E-cadherin-medicated cell-cell adhesion	69
			invasion	
	ND	ARFGEF2	E-cadherin and beta-catenin transport from the Golgi to cell surface	70
alpha-actinin	ARF1		interaction with complex of PLD2 and alpha-actinin	81
	ARF6	PSD	binding to alpha-actinin	84
beta-1 integrin	ARF6	PSD	protrusion formation	90
			recycling of beta-1 integrin, plakoglobin to PM	
	ARF6		recycling of beta-1 integrin to PM	93
			membrane ruffling	
			cell migration	
	ARF6	IQSEC1	internalization of beta-1 integrin	94
	ARF6	CENTB1	recycling of beta-1 integrin to the PM	96, 98
	ARF1, 3 ?	ARFGEF1	beta-1 integrin glycosylation	100
filamin A	ND	ARFGEF2	Filamin A transport from the Golgi to cell membrane	101
paxillin	ARF1		paxillin recruitment to FA	82
FA			actin stress fiber assembly	
	ARF1	DDEF1	paxillin localization	104, 107, 108
			cell spreading, migration	
			FA formation	
			membrane ruffling	
	ARF1, 5	DDEF1	binding to Src	110
	ARF6	DDEF2	paxillin recruitment to FA	105
			cell migration	
	ARF6	CENTD1	FA dynamics	106
	ARF6	GIT1, ARNO	binding to paxillin	114
			cell migration	
	ND	DDEF1	FA assembly	83
			binding to FAK and paxillin	
			cell spreading	
	ND	DDEF1	binding to paxillin and cortactin	109
			invasive activity	
	ND	GIT1	binding to PAK and PIX	111
			FC disassembly	
			cell motility	
	ND	GIT2	binding to paxillin and PIX	112
			actin remodeling	
	ND	GIT2	binding to paxillin	113
			membrane ruffling	
			FA turnover	
			cell spreading	

ND: not defined or known

fundamental cellular processes including secretion, endocytosis, cell adhesions and epithelial integrity. In Table 3, I have summarized the known activities of ARFs in functional integrity of epithelia. In addition, we are beginning to unravel their involvement in processes such as wound healing, cellular transformation, tumor cell invasion and metastasis. One challenge has been to understand the complex and intricate mechanisms that regulate these ARF proteins spatially and temporally. The circuitry of diverse GEFs and/or GAPs and their regulation by upstream signaling remains incomplete. Thus, ARF proteins continue to represent a very fertile and exciting field for us to make new discoveries and expand our understanding of these versatile molecules from basic science to clinical medicine.

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Abbreviations: ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factors; GAP, GTPase-activating proteins; TJ, tight junction; AJ, adherens junctions; FA, focal adhesions/contacts; BFA, brefeldin A; MDCK, Madin-Darby canine kidney; HGF, hepatocyte growth factor; EGF, epidermal growth factor; PI(4,5)P2, phosphatidylinosital (4, 5)-bisphosphate; PLD, phospholipase D; PIP5K, type I phosphatidylinositol phosphate 5 kinase; MHC I, major histocompatibility complex class I; ECM, extracellular matrix; FAK, focal adhesion kinase FAK; PAK, p21-GTPase-activated kinase; PIX, PAK-Interactive exchange factor; AIF, aluminum fluoride

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