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Requirement for Arf6 in Cell Adhesion, Migration, and Cancer Cell Invasion

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Cell movements are essential to life, in a variety of aspects including development, repair and defence processes. Cell migration is a multifactorial process in which a number of distinct events occur simultaneously. Besides its strong appeal towards the basic sciences, the molecular mechanisms of cell migration have long been major targets of oncology, including clinical studies aiming for cancer therapy and prevention. For the further advancement of these studies, as well as for the benefit of its clinical applications, it is important to understand the fundamental machinery and mechanisms regulating cell adhesion and motility. Here the possible roles of a small GTP-binding protein, Arf6, in epithelial cell adhesion and migration, and also in cancer cell invasion, are discussed.

Key words: Arf6, cadherin, cell migration, integrin, membrane recycling and remodeling.

Abbreviations: AJ, adherens junction; AP-2, adaptor protein-2; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; HGF, hepatocyte growth factor; IL-2R, interleukin 2 receptor; MHC, major histocompatibility complex; TfR, transferrin receptor.

As is well-known in the case of cancer cell invasion and metastasis, cell migration, when functioning abnormally, often threatens life. More than 80% of human tumors are of epithelial origin. Thus, efforts to study epithelial cell migration and to find the key event(s) regulating cell motility are under way, and are making extensive and fruitful progress. Much of the appeal of studying cell motility also arises from its essential roles for multicellular life.

Crawling animal cells seem to have a limited repertoire of movements. In general, they must first extend the leading edges of their plasma membranes, into the direction of movement. Such an extension process operates primarily *via* plasma membrane remodeling and cortical cytoskeletal remodeling.

Arf proteins belong to the Ras-superfamily of GTPases and are primarily involved in membrane trafficking and remodeling (*1*, *2*). Arfs are also extensively involved in the processes of cytoskeletal remodeling. The activity of Arf6, an Arf isoform, appears to be essential for efficient cell migration (*3*), while the activities of other Arfs such as Arf1 may be less closely involved in the fundamental aspects of cell migration (*4*). This short review discusses the roles of Arf6 in cell adhesion and migration. Our recent results showing its involvement in cancer cell invasion are also briefly mentioned.

Basic roles of Arf6 in endocytosis and recycling of plasma membranes

The Arf superfamily includes six isoforms of Arfs and several Arf-like proteins in mammalian tissues. Arf isoforms are classified into three classes by their structural similarities: class I (Arf1-3), class II (Arf4 and 5) and class III (Arf6). Arf1, the most thoroughly studied isoform, has been shown to regulate membrane traffic at multiple sites within the cell, especially in the perinuclear area. Arf6 is also engaged in membrane trafficking and remodeling, and functions primarily in membrane endocytosis and recycling at the cell periphery, probably through its GTPase cycle (*5*–*11*). The GTP-hydrolysis-defective mutant of Arf6, Arf6(Q67L), is mainly found localized to plasma membrane invaginated pits or accumulated in intracellular vacuoles, and acts to block the endocytosis of several cell surface molecules. On the other hand, the GTP-binding-defective mutant of Arf6, Arf6(T27N), has been shown to localize mainly to intracellular tubulovesicular structures, which are thought to represent the pericentriolar recycling compartment, and can block cell surface molecules from recycling back to the cell surface. The majority of endogenous Arf6 is found at the plasma membrane and intracellular compartments in the pericentriolar region (*9*, *12*). It has hence been proposed that Arf6, when loaded with GTP, may serve to regulate the outward flow of recycling membranes. Hydrolysis of GTP is then thought to signal the return of Arf6 and plasma membrane components to the tubular endosome. It should be noted, however, that these results were obtained primarily from observations using HeLa cells, but Arf6 may function differently in different cellular contexts (see below). Arf6 is widely expressed (*12*) and has been shown to function in a variety of biological events, including chromaffin granule exocytosis (*13*), Fcγ receptor-mediated phagocytosis (*14*, *15*), insulin stimulation (*16*), endocytosis at the apical plasma membrane (*17*), epithelial cell migration (*3*, *18*) and adherens junction (AJ) turnover (*18*). It is also involved in mechanisms other than membrane remodeling, such as actin cytoskeletal remodeling (*7*, *10*, *19*–*21*) and activation of phosphatidylinositol 5-kinase (*22*).

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Possible modes of Arf6 involvement in internalization and recycling of cell surface receptors

Besides plasma membrane components, Arf6 is involved in endocytosis and recycling of several types of receptors present on the plasma membrane. However, only a few types of cell surface receptors have been shown to be regulated by Arf6; and the issue of what types of receptors are under the control of Arf6 activity is still controversial and may be cell type-specific. It was first reported that Arf6 is involved in the recycling of transferrin receptors (TfR), which was assessed using CHO cells overexpressing human TfR at a high level (*5*). In this case, it was shown that both Arf6(Q67L) and overexpressed wild-type Arf6 decrease the rate of the endocytosis of transferrin, while Arf6(T27N) inhibits transferrin from recycling back to the cell surface. Overexpression of Arf1 had almost no effect on these activities. Partial colocalization of TfR and Arf6 was also observed by ultrastructural investigation in the same CHO cells (*9*). Internalization of TfR is a clathrin-dependent process. On the other hand, experiments using HeLa cells have shown that Arf6 might not be involved in clathrin-dependent endocytosis including that of the TfR, but is involved in endocytosis and recycling of major histocompatibility complex class I (MHC I) and Tac molecules, both of which apparently do not contain cytoplasmic tails conferring clathrin/AP-2 localization (*7*, *8*, *11*, *23*). Exogenously expressed Tac molecules were primarily used in these studies. Tac is a subunit of the interleukin 2 receptor (IL2R) (*24–26*), which in its active form is composed of the α (Tac), β and γ subunits (*27*). Tac exhibits very low affinity to IL2, and the biological significance of the internalization of Tac molecules, not integrated into the functional IL2R, remains elusive, except for the hypothesis that it may simply be a result of the basal turnover of left-over plasma membrane receptors (*26*). As mentioned earlier, Fcγ receptor-mediated phagocytosis in macrophages has also been demonstrated to be regulated by Arf6 activity, but not by the other Arfs (*14*, *15*). It is thought that phagocytosis is distinct from clathrin-mediated endocytosis (*28*), although there is a report suggesting direct involvement of clathrin in phagocytosis (*29*). Perry *et al.* have demonstrated that clathrin-coated pit-associated proteins are required for both opsonic and non-opsonic phagocytosis in alveolar macrophages (*28*). They also suggested, however, that clathrin-coated pit-associated proteins may not be a direct component of the phagocytic machinery, but may be required for endocytic recycling, which is perhaps necessary for membrane mass supply for pseudopod extension during phagocytosis.

The apparent function of Arf6, therefore, appears to be dependent on the cell type and cellular context. Similarly, it has also been suggested from the analysis of the expression pattern of Arf6 that it may function differently in different tissues and cells (*12*). Hence the molecular mechanims by which Arf6 exerts its different functions, depending on different cellullar contexts, remain to be clarified. Further identification of bona fide cell surface molecules that are directly under the control of Arf6 might also be necessary to establish the precise role of Arf6 in receptor endocytosis and recycling (also see below).

Involvement of Arf6 in the cellular dynamics of integrins and cadherins

Integrins are crucial for cell migration. Several types of integrins, such as the fibronectin receptors, are recycled and can be brought to the leading edges of the cell periphery in actively, migrating cells, although not all types of integrins enter recycling endosomes (*30–33*). Brown *et al.* have shown that overexpression of Arf6(Q67L) in HeLa cells can trap integrin β1 into the Arf6(Q67L)-enriched large vacuoles, which were found in a significant population of the Arf6(Q67L)-expressing cells (*11*). Other proteins such as MHC I and plakoglobin (γ-catenin), which may traffic through the Arf6 compartment, were also found enriched in such valuoles, while TfR was absent from these vacuolar structures. Consistent with the notion that GTP-Arf6 is an activator of phosphatidylinositol 4-phosphate 5-kinase (*22*), phosphatidylinositol 4, 5-bisphosphate was also highly enriched in such Arf6-enriched vacuoles. These observations hence provide evidence supporting that Arf6 activity may be involved in the intracellular dynamics of integrin β1. Whether the integrin β1 molecules in such vacuoles form complexes with some α subunits has not yet been addressed.

Cadherins are also endocytosed and perhaps recycled (*34*, *35*). Intracellular E-cadherin was, in part, found colocalized with Rab5-positive structures of early endosomes, while no clear colocalization was observed with Rab7-positive structures of late endosomes (*35*). Endocytosis of E-cadherin in MDCK cells is thought to occur through clathrin-mediated pathways, which was suggested from experiments using potassium depletion combined with hypotonic shock (*35*). This method was originally used to show the specific inhibition of clathrin-coated pit uptake of the low density lipoprotein receptors (*36*). Le *et al.* also confirmed that internalization of TfR was blocked by this treatment in MDCK cells, while the uptake of ricin, which occurs via a clathrin-independent mechanism, was unaffected (*35*). However, care should be taken with the evaluation of results obtained by this treatment, because potassium depletion effectively blocked phagocytosis of airway epithelial cells, in which clathrin may not be directly involved, while it was simultaneously shown that pinocytosis was almost unaffected (*37*). Moreover, potassium depletion can also abrogate some cellular functions, such as directional polarity in migrating fibroblasts (*38*). Involvement of Arf6 in the subcellular localization of E-cadherin has been reported, although how directly Arf6 is involved in the cellular dymanics of E-cadherin has yet to be analysed (*18*, see below).

Roles of Arf6 in cell migration

Palacios *et al.* have reported that Arf6 is involved in AJ turnover in polarized MDCK cells and regulates the spatial distribution and trafficking of E-cadherin as well as the cadherin-based junctional components, while Arf6 does not perturb the tight junctions (*18*). The effect of Arf6-GTP on AJ disassembly appears to be *via* vesicle trafficking, but is independent of actin remodeling and phospholipid metabolism. Palacios *et al.* also demonstrated that activation of Arf6 is required for haptotactic cell migration and the hepatocyte growth factor (HGF)-induced scattering activity of polarized MDCK cells, which is likely due to the disassembly of the AJs rather

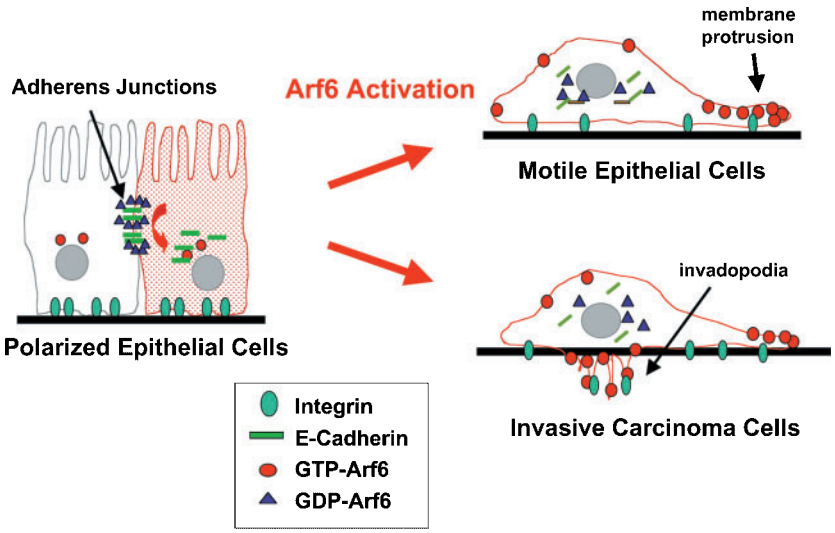

Fig. 1. **Requirement for Arf6 in cell adhesion, migration and cancer cell invasion.** Arf6-GDP is localized to cell-cell junctions in polarized epithelial cells, and its activation, to the GTP-bound form, acts to disassemble the junctions and inactivation of E-cadherin. Arf6 activity is then required for motile phenotype of epithelial cells, and for invasiveness of caricinomas. See text for in details.

than directly affecting the integrin-mediated migratory machinery. Activation of Arf6 by HGF treatment of MDCK cells has also been shown biochemically (*39*). Assembly of cell-cell junctions other than AJs, such as tight junctions and desmosomes, is dependent on the cadherin adhesions (*40*), and hence it was speculated that the disassembly of E-cadherin-based AJs may lead to the disassembly of other junctions in polarized MDCK cells (*18*). In these polarized MDCK cells, Arf6(Q67L) localizes to the basolateral membrane and cytoplasm, while Arf6(T27N) is localized predominantly to the AJs (*18*); both are opposite to those observed in isolated ("non-polarized") HeLa and CHO cells, as described earlier.

Overexpression of an Arf6GAP, AMAP2/PAG3/Papα, in Cos7 and in 12-*O*-tetradecanoylphorbol-13-ester-treated monocyte U937 cells effectively blocked haptotactic cell migration towards different extracellular matrices (*3*). On the other hand, overexpression of an Arf1GAP, Git2, did not immediately block cell motility (*4*). There is a discrepancy between the isoform specificity of AMAP2 measured biochemically *in vitro* and that measured cell biologically *in vivo* (*3*, *41*). A hypothesis to explain this discrepancy and describe the possible cellular mechanism of AMAP2 function has already been proposed (*42*). There are striking similarities in the processes for the remodeling of cytoskeletal architecture and membrane structure between the extension of pseudopods during phagocytosis and the extension of leading edges during cell migration (*43*). Arf6 is essential for Fcγ receptor-mediated phagocytosis at the initial stage of pseudopod extension, while the other isoforms are dispensable (*14*). AMAP2 expression is induced during monocyte maturation (*3*) and localized to the phagocytic cups (*15*). Uchida *et al*., have shown that AMAP2, when overexpressed, blocks Fcγ receptor-mediated phagocytosis in macrophages, also at the initial stage of pseudopod extension,

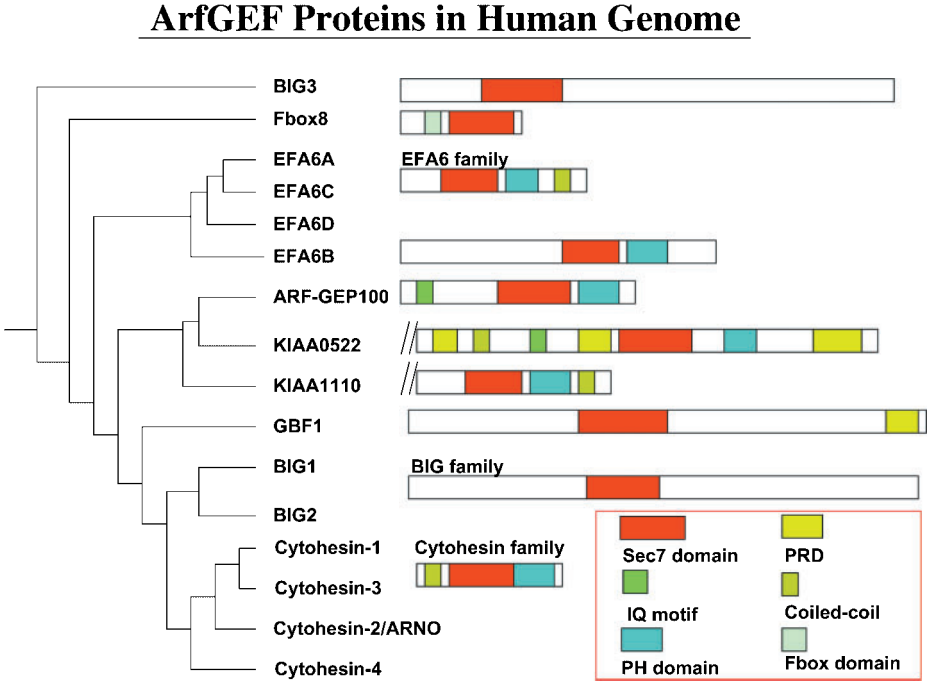

Fig. 2. **ArfGEF proteins in human genome.** Proteins bearing the *Sec 7* domain for putative ArfGEF activity are shown. For ArfGAP proteins in human genome, see Ref. *42*.

by antagonizing Arf6 (*15*). Git2 did not exhibit such blockage. It can therefore be speculated that overexpressed AMAP2 blocks membrane extension at the leading cell edges and hence blocks cell migration, although this notion needs to be addressed more precisely. We have more recently demonstrated that Arf6(Q67L) and Arf6(T27N) both block the haptotactic activity of Cos7 cells (S. Hashimoto and H. S., unpublished observations), in which the levels of mutant expression were carefully tuned to minimize their potential harmful effect on cell viability. The fact that both forms of the Arf6 mutants inhibit cell motility suggests the necessity of continued GTPase cycling of Arf6 in cell migration (see below).

Prospects

The functions of Arf6 in epithelial cell migration, described separately above, do not conflict but appear to be consistent with each other, with the possible following scenario (also see Fig. 1). For polarized epithelial cells, which form tight cell-cell adhesions, it is necessary to first disassemble the AJs to intiate migration, as has been described (*18*). In this step, the activation of Arf6 to Arf6-GTP is required. After being released from the tight adhesions, cells then still require Arf6 activity to extend the plasma membrane in the forward direction, for which continued recycling of the Arf6 GTPase between Arf6-GTP and Arf6-GDP to mediate endosomal recyling is required for membrane mass supply for the extension. A long-standing hypothesis proposed by Bretscher suggests that endosomal recycling plays an essential role in cell migration (*44–46*). Therefore, Arf6 may be considered as one of the factors regulating endosomal recycling that is closely coupled with cell motile activity, and hence as a central player in regulating cell motility. If such Arf6-regulated vesicles carry certain types of integrins, being engaged in cell adhesion and migration, Arf6 will then turn out to play an additional basic role in cell migration. Moreover, given that Arf6 plays a central role in cell motility, the mechanism for shutting off Arf6 activity must be considered to be fundamental for the stopping of cell migration and for contact inhibition of movement, as has also been suggested regarding endosomal activity in cell migration (*44*). The stopping of cell migration is of fundamental importance for tissue morphogenesis as well as neuronal network formation.

Tumor invasion of epithelial cancers often occurs through collective migration, rather than the migration of individual cells (*47*). Carcinoma cells bearing high metastatic potential, in most cases, have already lost normal E-cadherin expression and localization, but sometimes express other cadherins such as N-cadherin (*48*). It has, moreover, been shown that forced expression of N-cadherin in noninvasive, E-cadherin-positive human breast carcinoma cells can produce an invasive cell (*49*). The mode of regulation of the intracellular dynamics and trafficking of E-cadherin and N-cadherin is not identical in epithelial cells, while Arf6 activity appears to be involved in their subcellular localization (K. Miura and H. S., unpublished observations). Therefore, further analyses on the roles of Arf6 may be required for the understanding of invasion and metastasis of epithelial cancers. If Arf6 is not directly involved in the control of the invasive cadherins, like N-cadherin, identification of the corresponding small G protein is required. We have recently found that Arf6 is enriched in invadopodia of metastatic human breast cancer cells, and shown that both invasive activity and migration of the breast cancer cells can be effectively blocked by an siRNA treatment specific for Arf6 (S. Hashimoto, Y. Mazaki and H.S., unpublished observations; also see Fig. 1). Moreover, we have identified an Arf6GAP specifically enriched in invadopodia of breast cancer cells (Y. Onodera and H.S., unpublished observations).

The number of genes for ArfGAP and ArfGEF in humans is significantly larger (more than two- to three-fold) than that for the Arf isoforms and Arf-like factors (Fig. 2; *42*; I. Kobayashi and H.S., unpublished observations). Further identification and detailed analysis of the GAPs and GEFs acting on Arf6 during each aspect of Arf6 function, coupled with siRNA-mediated inhibition of their protein expression, might greatly contribute to understanding the essential mechanisms regulating cell motility, cancer cell invasion and metastasis.

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