

Cell migration: GAPs between membrane traffic and the cytoskeleton

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During cell migration, coordination between membrane traffic, cell substrate adhesion and actin reorganization is required for protrusive activity to occur at the leading edge. Actin organization is regulated by Rho family GTPases and, with a contribution from the endocytic cycle, serves to extend the cell front. The details of the molecular mechanisms that direct membrane traffic at sites of adhesion and rearrange actin at the cell edge are still unknown. However, recent findings show that a number of multi-domain proteins characterized by an ArfGAP domain interact with both actin-regulating and integrin-binding proteins, as well as affecting Rac-mediated protrusive activity and cell migration. Some of these proteins have been shown to localize to endocytic compartments and to have a role in regulating endocytosis. Given the participation of Arf proteins in regulating membrane traffic, one appealing hypothesis is that the ArfGAPs act as molecular devices that coordinate membrane traffic and cytoskeletal reorganization during cell motility.

Membrane traffic and actin dynamics at the leading edge

Cell migration is driven by the protrusive activity at the leading edge of the cell, where continuous remodelling of actin and adhesive contacts is required. It has been hypothesized that membrane internalized from the cell surface is recycled to the front of migrating cells to contribute to the extension of the cell border (Bretscher, 1996). Given the rapid rate of membrane internalization (Hao and Maxfield, 2000), large amounts of recycling membrane would be made available for polarized delivery by such a mechanism. Consistent with this model, recycling transferrin receptors and low density lipoprotein receptors are distributed to the cell front of migrating fibroblasts and to Rac-induced ruffles (Hopkins *et al.*, 1994; Bretscher and

Aguado-Velasco, 1998a). Thus, the random reinsertion of internalized membranes at the surface of a resting cell may be redirected to the sites of protrusion when migration is induced by motogenic stimuli (Bretscher and Aguado-Velasco, 1998b).

While our knowledge of the molecular machinery underlying the propulsive mechanism driven by actin and mediated by Rho family GTPases has increased dramatically (Hall, 1998; Borisy and Svitkina, 2000), it is still unclear how, and to what extent, vesicle recycling is incorporated into the extension process. Progress in this direction, however, comes from studies of Arf6, a member of the ADP-ribosylation factor (Arf) family of GTPases. This protein has been implicated in the regulation of membrane traffic between the recycling endosomal compartment and the plasma membrane because of the specific localization of Arf6 in these compartments, and the effects of its overexpression on transferrin uptake and recycling to the cell surface (D'Souza-Schorey *et al.*, 1995; Peters *et al.*, 1995). Moreover, the Arf6-positive intracellular compartment overlaps with the transferrin receptor-positive recycling compartment (D'Souza-Schorey *et al.*, 1998). Based on the fact that Arf1 regulates specifically the formation of vesicles within the Golgi compartment (Roth, 1999), one could speculate that Arf6 would also regulate vesicle formation, in this case during recycling between endosomes and the plasma membrane. Arf6 appears to be functionally linked to Rac1, a Rho family GTPase involved in the formation of actin-rich ruffles and lamellipodia (Ridley *et al.*, 1992). Rac1 and Arf6 colocalize at the plasma membrane and on recycling endosomes, and Rac1-stimulated ruffling is blocked by the GTP binding-defective N27-Arf6 mutant (Radhakrishna *et al.*, 1999). Together, the data regarding Arf6 involvement in vesicle trafficking and the association with Rac1 have led to the suggestion that the ability of Arf6 to influence Rac1-mediated lamellipodial formation depends in part on Arf6-mediated regulation of Rac1 trafficking to the plasma membrane.

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I. de Curtis

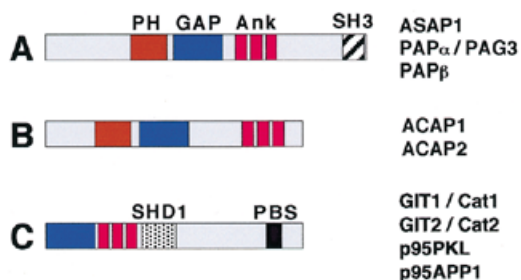


Fig. 1. Schematic representation of three recently identified groups of multi-domain proteins characterized by the presence of an ArfGAP domain (GAP) and by ankyrin repeats (Ank). (A) ASAP1 and two PAP proteins are characterized by the presence of a pleckstrin homology domain (PH) and a C-terminal Src homology type 3 (SH3) domain. (B) ACAP proteins, include a PH domain. (C) Four members of the GIT family are characterized by a Spa2 homology 1 domain (SHD1) (Sheu *et al.*, 1998), and a C-terminal paxillin binding subdomain (PBS). Proteins in (A) and (B) are members of the centaurin family, which is characterized by ArfGAP domain, ankyrin repeats and PH domain.

The question left largely unsolved relates to what the mechanisms underlying the postulated recycling of membrane to polarized sites of actin organization are. The focus here is on recent findings on a group of proteins that share an Arf-specific GAP (GTPase-activating protein) domain and are thus implicated in the coordination between membrane trafficking and actin reorganization during cell locomotion.

More than just GAPs: a connection

Arfs cycle between the GTP- and GDP-bound forms with the help of specific GAPs and GEFs (guanine nucleotide exchange factors) (Donaldson and Jackson, 2000). Recently, a number of multi-domain proteins with an ArfGAP domain have been identified (Figure 1) and these are capable of interacting with proteins involved in both cell adhesion and actin organization. Given the proposed role of Arf6 in membrane recycling, one attractive hypothesis is that the ArfGAP activity of some of these proteins is required for the regulation of Arf-mediated membrane recycling to sites of protrusion during cell locomotion.

Support for this hypothesis comes from recent data on ArfGAPs of the centaurin family. Two of these, ASAP1 and PAP α (Figure 1A), show *in vitro* GAP activity toward Arf1, Arf5 and, to a lesser extent, Arf6. They interact with Src and Pyk2, respectively (Brown *et al.*, 1998; Andreev *et al.*, 1999), two tyrosine kinases that have been implicated in the regulation of integrin-mediated adhesion (Schlaepfer and Hunter, 1998). ASAP1 localizes to peripheral focal complexes, and requires a functional ArfGAP domain for actin remodelling in motile cells (Randazzo *et al.*, 2000). PAP α -paxillin complexes are recruited to the cell periphery. Paxillin is a focal adhesion scaffolding protein (Turner *et al.*, 1990), which has been proposed to play a role in focal adhesion dynamics. Overexpression of wild-type PAP α , but not of a GAP-inactive mutant form, inhibits paxillin recruitment to focal contacts and sites of cell migration, resulting in decrease of the cell migratory activity on extracellular matrix (Kondo *et al.*, 2000). The data support the hypothesis that the recruitment of both structural (paxillin) and signalling (Src and Pyk2) molecules

to the leading edges of migrating cells, a step that favours the formation of new adhesive contacts at these sites, is not mediated by simple cytoplasmic diffusion, but rather through these ArfGAPs. Two other centaurins, ACAP1 and ACAP2 (Figure 1B), show strongest GAP activity toward Arf6 (Jackson *et al.*, 2000). Overexpression of ACAPs prevents the formation of Arf6-dependent protrusions and leads to redistribution of ACAPs to endosomal structures, together with activated Arf6. Again, a functional GAP domain is required for these effects, implicating the GAP activity of ACAPs in the regulation of Arf6-mediated membrane recycling from the endosomal compartment (where Arf6 is activated to Arf6-GTP) (Radhakrishna and Donaldson, 1997) back to the plasma membrane.

The GIT family of ArfGAPs (Figure 1C) includes multi-domain proteins with an N-terminal ArfGAP domain (Premont *et al.*, 1998). These exhibit *in vitro* GAP activity toward several Arfs, including Arf6 (Vitale *et al.*, 2000). GIT proteins are components of complexes (Figure 2) which may also include the GEF PIX (Oh *et al.*, 1997; Bagrodia *et al.*, 1998; Manser *et al.*, 1998), the Rac effector PAK (Daniels and Bokoch, 1999), and the adaptor protein Nck (McCarty, 1998; Turner *et al.*, 1999). Existing data support the hypothesis that GIT proteins play a role in Arf6-mediated membrane recycling. It is known that GIT proteins can affect endocytosis (Claing *et al.*, 2000), and dissection of these multi-domain proteins has been useful in identifying possible distinct functions for individual domains. For example, the N-terminal region of p95APP1 colocalizes with the GTP-binding defective mutant N27-Arf6 at endocytic vesicles (Di Cesare *et al.*, 2000), where this inactive GTPase accumulates. The first ankyrin repeat of this protein is necessary for membrane targeting of the ArfGAP domain, which by itself is cytosolic (Di Cesare *et al.*, 2000). Another possible link of GIT proteins to membrane recycling is the partial localization of p95APP1 to large, transferrin receptor-positive endocytic vesicles (Di Cesare *et al.*, 2000), which may represent a functionally altered recycling compartment. This localization is strongly enhanced by co-expression of PIX, and also for a truncated form of p95APP1 that includes the SHD1 PIX-binding domain but lacks the N-terminal region in which the ArfGAP domain resides (Di Cesare *et al.*, 2000). These data point to a role for the SHD1 domain in PIX-mediated recruitment to the recycling compartment. It can be speculated that the resulting lack of the GAP activity at vesicles would interfere with Arf6-mediated membrane recycling, leading to the accumulation of internalized membrane in an abnormal perinuclear recycling compartment.

Most GIT proteins interact with paxillin by their C-terminal region (Turner *et al.*, 1999). A p95APP1 C-terminal paxillin-binding construct strongly enhances the protrusive activity at the cell edge in a Rac- and Arf6-dependent manner, with relocation of paxillin to the sites of protrusion (Di Cesare *et al.*, 2000). Overexpression of GIT1 causes loss of paxillin from existing focal complexes and stimulates cell motility (Zhao *et al.*, 2000b), while inhibition of the interaction between paxillin and p95PKL prevents lamellipodial formation (Turner *et al.*, 1999). Interestingly, PIX-mediated recruitment of p95APP1 to the large endocytic vacuoles is accompanied by recruitment of paxillin from older focal complexes to the same vesicles (Di Cesare *et al.*, 2000). Consistent with this finding, the severe paxillin reduction observed at focal adhesions in response to GIT1 overexpression is accompanied by the localization of paxillin at large perinuclear

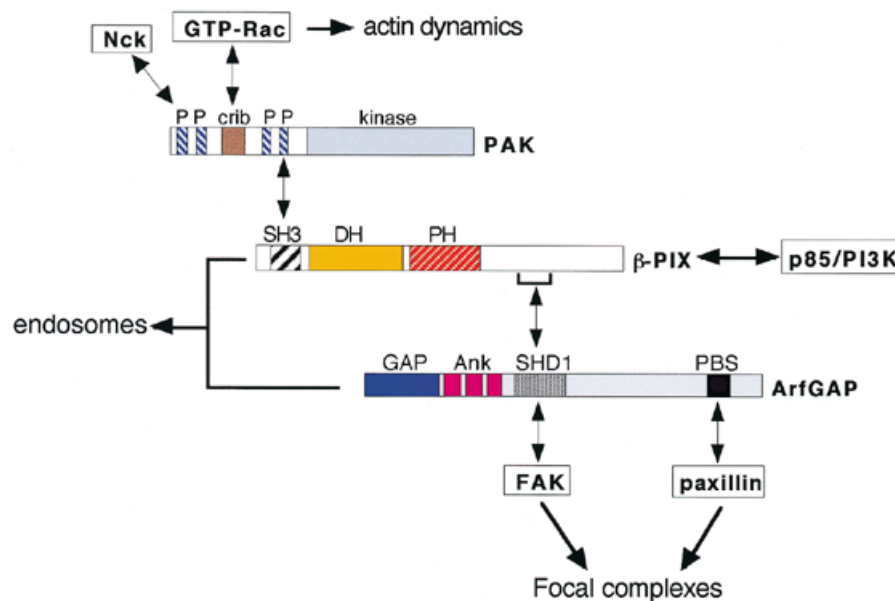


Fig. 2. Model for the intermolecular interactions and functional connections proposed for members of the GIT family (Figure 1C). Double-ended arrows point to known direct intermolecular interactions; single-ended arrows indicate functional connections. P, Proline-rich region; crib, Cdc42/Rac interactive binding motif; SH3, Src homology type 3; DH, Dbl homology; PH, pleckstrin homology; GAP, ArfGAP domain; Ank, ankyrin repeat; SHD1, Spa2 homology domain type 1; PBS, paxillin binding subdomain; p85/PI3K, 85 kD regulatory subunit of the phosphatidylinositol-3 kinase; ArfGAP = multi-domain ArfGAP protein. Members of the GIT family of ArfGAP proteins (indicated as ArfGAP) can stably interact with PIX and PAK (Bagrodia *et al.*, 1999; Turner *et al.*, 1999; Di Cesare *et al.*, 2000), which mediate the interaction of the complex with active Rac and Cdc42 at the membrane. The complex may regulate actin remodelling at the cell surface by controlling Rac/Cdc42 activity via PAK and PIX. The interaction of ArfGAP proteins with paxillin (Turner *et al.*, 1990, 1999) and FAK (Zhao *et al.*, 2000b) functionally links the complex to integrin-mediated adhesion. Finally, the ArfGAPs are localized to the endosomal compartment by both PIX-dependent and PIX-independent mechanisms.

vesicles (Zhao *et al.*, 2000b). Altogether, these data indicate a possible connection between paxillin and membrane recycling, and implicate the GIT-paxillin complexes in the protrusive activity.

According to the model proposed here (Figure 3), GIT proteins would form stable complexes linking Rho and Arf family GTPases by cycling between the endocytic recycling compartment and the plasma membrane. This mechanism would provide a way to deliver to the cell edge both membranes and the molecules required to stimulate actin polymerization and the formation of new adhesive sites. On one hand, GIT-paxillin complexes would affect adhesion and actin organization by recruiting paxillin to new Rac-induced focal complexes at the cell edge (Nobes and Hall, 1995). On the other, GIT complexes would position PIX and PAK at the cell border to regulate Rac function (Daniels and Bokoch, 1999). In this picture, the ArfGAP activity would be part of the engine required to efficiently re-utilize the endocytosed membrane for cell motility.

How are vesicles budding from the recycling compartment targeted to the migrating cell edge? Recent results have indicated that PIX may be recruited to the membrane either via the formation of a complex with PAK and Nck, or by direct association with the p85 regulatory subunit of PI3-kinase (Yoshii *et al.*, 1999). During cell motility, the localized activation of adhesive or guiding receptors by extracellular cues would drive anchoring of the PIX-ArfGAP complex at new sites by one of these mechanisms. As a consequence, in addition to the stimulation of the PAK

kinase activity by PIX, PAK would stimulate the exchange factor activity of PIX (Daniels *et al.*, 1999). Alternatively, the localization of the ArfGAP-PIX-PAK complex could be driven by PAK to areas of the plasma membrane that are enriched in GTP-Rac (Figure 3), which, in turn, might be recruited in response to extracellular adhesive and motogenic stimuli (del Pozo *et al.*, 2000).

Perspectives

At this stage, the proposed function of the multi-domain ArfGAP proteins represents mostly a working model, with several open questions to be answered. Several aspects of cell biology will need to be merged to address the issue in a complete way. Among the requirements are a better characterization of the intracellular recycling compartments involved, and of the molecular machinery linking these complexes to the endosomal membranes. It will also be necessary to identify the Arf proteins targeted by all these ArfGAPs *in vivo*, as well as to demonstrate the role of the ArfGAP activity in membrane recycling in cell-free systems.

Also worthy of attention is the apparent incongruence between the localization of some of these ArfGAPs to the endosomal compartment, and the reported accumulation of active Arf6 mutants at the plasma membrane where known Arf6 GEFs, such as ARNO (Frank *et al.*, 1998) and EFA6 (Franco *et al.*, 1999), have also been shown to reside. One possibility is that

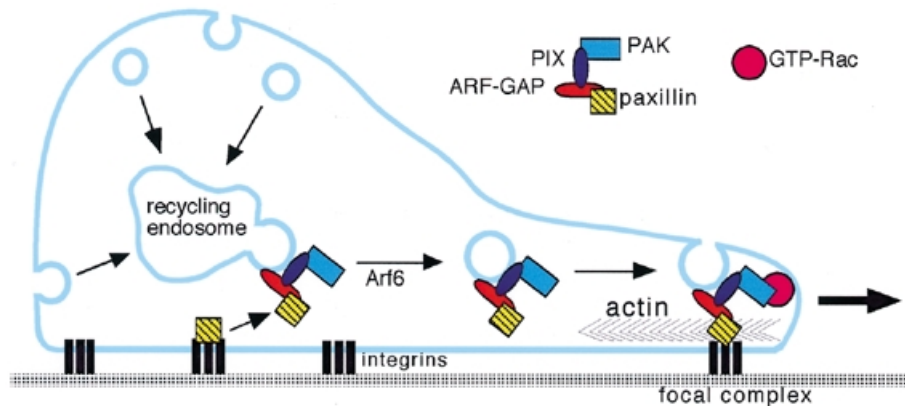


Fig. 3. Proposed model for the role of multi-domain ArfGAPs of the GIT family during cell migration. The ArfGAP protein is able to colocalize with Arf6 in the recycling endosomal compartment via its ankyrin repeats and the interaction with PIX. There the internalized membranes converge before recycling. In the proposed model it is speculated that wild-type Arf proteins (including Arf6) and the GAP activity of the ArfGAP are required for the formation of recycling vesicles. Once formed, vesicles may be recruited to Rac-enriched sites of the leading edge of the migrating cell. By recruiting paxillin to the complex, ArfGAP proteins would induce the redistribution of paxillin away from established focal adhesions to the leading edge. Here, paxillin would contribute to the formation of membrane protrusions, by participating in the formation of focal complexes in which paxillin is required for the anchorage of the Rac-induced actin filaments to the sites of substrate adhesion. Regulatory mechanisms must exist to direct distinct pools of the complex to endosomal membranes or to the cell surface.

the substrate-independent subcellular localization of these proteins represents a way to limit their catalytic activity to sites where endogenous Arf6 needs to be regulated to perform its specific functions, and these sites may not be reflected by the distribution of Arf6 mutants as revealed by morphological analysis. Further work will be required to unravel this issue.

In view of the dynamic nature of the mechanisms in which the ArfGAP complexes are implicated, it is not surprising that their regulation within the cell appears to be extremely complicated. Examples of functional regulation include the PIX-dependent interaction of GIT1 with paxillin (Zhao *et al.*, 2000b), the PIX-mediated localization of PAK to focal complexes (Manser *et al.*, 1998) and the disruption of the PIX-PAK complex as a consequence of PAK activation (Zhao *et al.*, 2000a). Further analysis of these regulatory mechanisms will be fundamental to understanding how these complex interactions may be dynamically and spatially coordinated during cell migration. Although at its primordial stages, an integrated analysis of the mechanisms that lead to polarized delivery of membranes during cell migration should finally lead us to understand, in a more comprehensive way, the fundamental process of cell motility.

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Ivan de Curtis

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