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Contribution of AZAP-Type Arf GAPs to Cancer Cell Migration and Invasion

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Abstract

Arf GAPs are a family of proteins with a common catalytic domain that induces hydrolysis of GTP bound to the small GTP-binding protein Arf. The proteins are otherwise structurally diverse. Several subtypes of Arf GAPs have been found to be targets of oncogenes and to control cell proliferation and cell migration. The latter effects are thought to be mediated by coordinating changes in actin remodeling and membrane traffic. In this chapter, we discuss Arf GAPs that have been linked to oncogenesis and the molecular mechanisms underlying the effects of these proteins in cancer cells. We also discuss the enzymology of the Arf GAPs related to possible targeted inhibition of specific subtypes of Arf GAPs.

I. INTRODUCTION

Carcinogenesis is a complex process involving changes in cell proliferation, apoptosis, migration, and adhesion. Signaling pathways controlling each of these cellular activities have been identified. The mechanisms by which the activities are coordinated are still being discovered. Arf GAP proteins, identified as regulators of Arf family GTP-binding proteins, are interfaces between signaling pathways. The Arf GAPs also function as scaffolds and have intrinsic activities, such as bending membranes, which may directly contribute to the aberrant behavior of cancer cells.

II. SIGNALS INFLUENCED BY Arf GAPs

Proliferative and migration signals that are influenced by Arf GAPs are initiated by receptor tyrosine kinases (RTKs). These are transmembrane proteins such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Blume-Jensen and Hunter, 2001; Hunter, 2000; Pawson and Scott, 1997; Schlessinger, 2000). In normal physiology, tyrosine kinase activity in these proteins is activated by ligand binding. At least seven polypeptide ligands, including epidermal growth factor (EGF) and transforming growth factor α (TGF α), bind to EGFR. The peptide platelet-derived growth factor (PDGF) binds to PDGFR. The receptors autophosphorylate, which creates binding sites for adaptor proteins and signaling proteins that contain SH2 and PTB domains. Proteins recruited to the membrane either by direct interaction with RTKs or indirectly include nonreceptor tyrosine kinases such as the oncogene Src, phospholipase C γ , phosphatidylinositol 3-kinase, and

exchange factors for Ras family GTP-binding proteins and Rho family GTP-binding proteins. Ras-GTP stimulates the MAP kinase pathway, leading to changes in transcriptional activity and, consequently, cell proliferation. Ras-GTP also stimulates PI3K, which generates the signaling lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 activates the serine/threonine kinase Akt, which inhibits apoptosis and stimulates protein synthesis and cell proliferation. RhoA-GTP, Rac1-GTP, and Cdc42-GTP are generated, which act through different classes of effectors to alter the actin cytoskeleton and change transcription leading to proliferation.

III. CELLULAR ADHESIVE STRUCTURES AFFECTED BY Arf GAPs

Cellular adhesive structures mediate cell movement and are involved in cellular signaling. At least three adhesive structures are affected by Arf GAPs (Fig. 1).

A. Focal Adhesions

Focal adhesions (FAs) are the best characterized of the adhesive structures affected by Arf GAPs (Weaver, 2006; Yamaguchi *et al.*, 2006). They are points of attachment of the actin cytoskeleton to the extracellular matrix (ECM). FAs contain transmembrane heterodimeric proteins called integrins. The extracellular portion of integrins bind to proteins in the ECM, such as fibronectin. The cytoplasmic part of integrin binds to bundles of actin filaments called stress fibers (Burridge and Chrzanowska-Wodnicka, 1996; Hynes, 1999, 2002). Other proteins associated with FAs include FAK, paxillin, vinculin, integrin-linked kinase, tensin, talin, and actinin.

FAs are dynamic structures that undergo a maturation process. Most of the components, excluding zyxin, are found in structures called focal complexes, at the edge of the cell. These mature, on a time scale of minutes, into FAs that appear as linear structures behind the edge of the cell at the end of actin stress fibers (Etienne-Manneville and Hall, 2002; Hall, 1998; Jaffe and Hall, 2005; Mackay and Hall, 1998; Ridley and Hall, 1992). FAs undergo further maturation to become more stable structures located more centrally in the cell. The process of maturation requires actin polymerization, stress fibers, and contraction of the stress fibers. Even in FAs that appear to be stable, the component proteins turn over on a time scale of under a minute.

B. Invadopodia and Podosomes

The other two adhesive structures affected by Arf GAPs are invadopodia and podosomes. Invadopodia, found in cancer cells, are sites of both attachment to and degradation of the ECM. Podosomes are highly related structures first identified in Rous-sarcoma virus-transformed fibroblasts (Buccione *et al.*, 2004; Davidpfeuty and Singer, 1980; Marchisio *et al.*, 1987; Tarone *et al.*, 1985; Weed and Parsons, 2001) and later reported to be present in cells of the monocytic lineage, such as macrophages, osteoclasts, and dendritic cells, and in epithelial, endothelial, and smooth muscle cells (Gimona, 2003; Gimona and Buccione, 2006; Marchisio *et al.*, 1984, 1987; Spinardi and Marchisio, 2006). Podosomes mediate migration and invasion, which is necessary, for instance, for the function of phagocytes. Invadopodia and podosomes contain integrins, cortactin, polymerized actin, and matrix

metalloproteinases. Invadopodia appear as puncta or small rings on the ventral surface of cells (Ayala *et al.*, 2006; Bowden *et al.*, 1999; Buccione *et al.*, 2004; Linder and Aeppelbacher, 2003; Spinardi and Marchisio, 2006; Weaver, 2006; Yamaguchi *et al.*, 2006). In podosomes, the puncta may coalesce to form rosettes (Buccione *et al.*, 2004; Linder and Aeppelbacher, 2003; Spinardi and Marchisio, 2006). Like FAs, podosomes and invadopodia are dynamic structures.

IV. THE SUBSTRATES FOR THE Arf GAPs: Arf FAMILY GTP-BINDING PROTEINS

ADP-ribosylation factor (Arf) family GTP-binding proteins are the substrates for Arf GAPs. Six genes encode Arf proteins in mammals, five in human (Donaldson, 2003; Donaldson *et al.*, 2005; Gillingham and Munro, 2007; Kahn *et al.*, 2006; Logsdon and Kahn, 2003; Moss and Vaughan, 1998; Souza-Schorey and Chavrier, 2006). Based on primary sequence, these have been divided into class 1 (Arf1, Arf2, and Arf3), class 2 (Arf4 and Arf5), and class 3 (Arf6) (Logsdon and Kahn, 2003; Moss and Vaughan, 1998). The name for these proteins comes from the pathologic activity for which they were identified: Arfs function as cofactors for cholera toxin-catalyzed ADP-ribosylation of the heterotrimeric G protein Gs (Kahn and Gilman, 1984, 1986). This activity does not appear to be related to the function of Arf proteins in normal physiology. Most work on Arf proteins in mammals has focused on Arf1 and Arf6. They regulate membrane traffic and the actin cytoskeleton and are involved in cell migration (Donaldson, 2003; Gillingham and Munro, 2007; Randazzo *et al.*, 2000a; Souza-Schorey and Chavrier, 2006).

The molecular basis for function in membrane traffic is best described for Arf1 (Nie and Randazzo, 2006; Nie *et al.*, 2003b; Randazzo *et al.*, 2000a; Rothman, 2002; Spang, 2002; Springer *et al.*, 1999) (Fig. 2). In the prevailing paradigm, Arf1-GTP binds to both membranes and coat proteins, recruiting coat proteins to the membrane surface. The coat proteins trap cargo, polymerize, and drive the formation of transport intermediates. Arf1-GTP is converted to Arf1-GDP by hydrolyzing GTP. Arf1-GDP does not bind either membranes or coat protein with high affinity. Consequently, the coat protein dissociates forming a transport intermediate that is competent to dock with and fuse to a target membrane. Arf1-GTP is able to interact with other proteins that may be effectors, such as PI 4-kinase (Godi *et al.*, 1999; Jones *et al.*, 2000; Krauss *et al.*, 2003; Nie *et al.*, 2003b; Randazzo *et al.*, 2000a). Activating this enzyme at the Golgi apparatus is necessary for transport through and maintenance of the Golgi stack.

The molecular basis for Arf6 control of membrane traffic is not strictly analogous to that for Arf1. The effect of Arf6 on membrane traffic and actin remodeling is mediated, at least in part, by enzymes that metabolize signaling lipids (Nie *et al.*, 2003b). Arf6 binds to and activates phospholipase D (Brown *et al.*, 1993; Cockcroft *et al.*, 1994), which hydrolyzes phosphatidylcholine to generate phosphatidic acid (PA). Arf6 also binds to and activates phosphatidylinositol (PI) 4-kinase and phosphatidylinositol 4-phosphate (PI4P)-5 kinase (Anderson *et al.*, 1999; Balla, 1998; Honda *et al.*, 1999; Jones *et al.*, 2000; Perez-Mansilla *et al.*, 2006), resulting in the production of phosphatidylinositol 4,5-bisphosphate (PIP2). PIP2

has regulatory functions and is a precursor for the signaling molecules PIP3, diacylglycerol, and inositol 1,4,5 trisphosphate. The effects of Arf6 on PA and PIP2 have been found to be necessary for Arf6-dependent membrane traffic and actin remodeling (Brown *et al.*, 2001; Cockcroft *et al.*, 1994; Fensome *et al.*, 1996; O’Luanaigh *et al.*, 2002). Arfaptin is another Arf6 effector (Cherfils, 2001; Kanoh *et al.*, 1997; Tarricone *et al.*, 2001; Williger *et al.*, 1999). Arfaptin is a BAR domain protein that binds to Rac·GDP. Rac·GDP is displaced by Arf6·GTP, which may be a mechanism by which Rac·GTP levels are locally regulated. Arf6 also binds to class 2 Rab11-binding proteins FIP3 and FIP4 (Fielding *et al.*, 2005; Hickson *et al.*, 2003; Horgan *et al.*, 2004, 2007). The molecular basis of action of FIP3 and FIP4 is still being discovered. Arf6 also regulates an ACAP1/clathrin coat, which controls the endocytosis of transferrin and cell adhesion molecules called integrins (Dai *et al.*, 2004; Li *et al.*, 2005, 2007).

Arf6 has been implicated in the invasive behavior of cancer cells. Arf6 associates with invadopodia in MDA-MB-231 cells, which is a cell line derived from an invasive mammary carcinoma (Hashimoto *et al.*, 2004; Sabe, 2003). Reducing Arf6 expression with siRNA had no effect on cell viability, but the cells were less migratory and invasive than controls. Dominant negative or constitutively active Arf6 or Arf1 also inhibited migration and invasion, whereas expressing analogous mutants of Arf5 had no effect. Similar results were obtained in a melanoma cell line (Tague *et al.*, 2004). Cells expressing constitutively active Arf6 were more invasive than controls, and cells expressing dominant negative Arf6 were less invasive. The level of invasiveness correlated with activation of Erk. Arf6 is activated downstream of the EGFR by the exchange factor BRAG2, which was required for EGF-induced migration and invasion of a mammary carcinoma cell line (Hiroi *et al.*, 2006; Morishige *et al.*, 2008). These results are consistent with a model in which Arf6, activated by a growth factor, regulates Erk. Erk regulates the formation of invadopodia.

The function of Arfs requires cycling between the GTP- and GDP-bound forms of the protein. Arfs, however, have very low exchange rates and no detectable intrinsic GTPase activity. The cycle of GTP binding and hydrolysis is dependent on accessory proteins called guanine nucleotide exchange factors (GEFs), such as BRAG2, which was described above, and GTPase-activating proteins called GAPs.

V. THE Arf GAP FAMILY

Thirty-one genes encoding proteins with the Arf GAP catalytic domain have been identified in humans (Kahn *et al.*, 2008; Fig. 3). Arf GAPs were first identified on the basis of the enzymatic activity of inducing the hydrolysis of GTP that is bound to Arf family GTP-binding proteins (Brown *et al.*, 1998; Cukierman *et al.*, 1995; Makler *et al.*, 1995; Randazzo and Kahn, 1994). Most Arf GAPs have multiple domains consistent with complex regulation and with multiple distinct functions (Inoue and Randazzo, 2007; Nie and Randazzo, 2006; Randazzo and Hirsch, 2004; Randazzo *et al.*, 2007). Phylogenetic analysis of the Arf GAP domains or categorization based on domain structure yields a similar subclassification of the family (see Fig. 3A and B) (Kahn *et al.*, 2008). The 10 major subtypes are called Arf GAP1-type, Arf GAP2/3, ADAPs, SMAPs, AGFGs, Gits, ASAPs, ACAPs, ARAPs, and AGAPs. The Arf GAP1, Arf GAP2/3, ADAP, SMAP, AGFG, and Git subtypes have the catalytic

domain at the extreme N-terminus of the protein. The ASAPs, ACAPs, ARAPs, and AGAPs have an Arf GAP domain sandwiched between PH and Ank repeat domains. Based on this feature, these four subtypes are sometimes called *AZAP* for Arf GAP, Ank repeat, and PH domains. The “Z” stands for additional domains identifying a particular subtype. SMAPs and Gits have been implicated in oncogenesis and are the subject of excellent reviews (de Curtis, 2001; Meyer *et al.*, 2005; Tanabe *et al.*, 2006; Turner *et al.*, 2001). We will focus on the AZAP group of Arf GAPs. The AZAPs have been, in some cases, directly implicated in oncogenesis (Ahn and Ye, 2005; Ehlers *et al.*, 2005; Liu *et al.*, 2007; Onodera *et al.*, 2005). Furthermore, they are the targets of oncogenes, mediate or influence signaling pathways that are often disrupted or usurped in cancer cells, and regulate cellular adhesive structures that contribute to cell migration and invasion.

Four Arf GAP subtypes are within the AZAP group: ASAPs, ACAPs, ARAPs, and AGAPs. Each has a number of alternate names as detailed in recent reviews and in a commentary on the nomenclature of Arf GAPs (Gillingham and Munro, 2007; Inoue and Randazzo, 2007; Kahn *et al.*, 2008; Table I). The nomenclature is based on the domain structure of the proteins that are the prototypes of each group. The ASAPs contain BAR, PH, Arf GAP, ankyrin repeat, and proline-rich SH3-binding motifs. ASAP1 contains a tandem repeat of E/DLPPKP. ASAP1 and ASAP2 also contain an SH3 domain, which is the basis for the “S” in the name, but ASAP3 does not. If the nomenclature is retained, “S” can stand for “SH3 binding site.” There are three ACAPs. They contain BAR, PH, Arf GAP, and ankyrin repeat domains. The “C” stands for a predicted coiled coil domain, which has now been identified as a BAR domain. There are three ARAPs, which contain SAM, 5 PH, Arf GAP, ankyrin repeat, Rho GAP, and Ras association domains. The “R” stands for Rho GAP. The AGAPs are the largest group with 11 members. They contain a GTP-binding protein-like domain and a split PH domain in addition to the Arf GAP and Ank repeat domains. The GTP-binding domain of AGAP1, for instance, has 28% identity with HaRas. The AGAPs and ASAPs have each been implicated in carcinogenesis. ARAPs and ACAPs have been implicated in cell behaviors important to cell migration and cancer-cell invasion and for cell signaling but have not been directly implicated in cancer.

VI. Arf GAP SUBTYPES IMPLICATED IN CARCINOGENESIS

A. AGAP Proteins in Glioblastoma

AGAPs were the first Arf GAPs implicated in cancer. Snyder and colleagues (Ye *et al.*, 2000) identified a rat protein that was a splice variant of AGAP2, called PIKE-S, in a two-hybrid screen for proteins that bound to 4.1 N, a neuronal membrane cytoskeleton protein that mediates the antimitotic actions of NGF. PIKE-S is identical to the N-terminal GTP-binding domain and part of the PH domain of AGAP2 but has a limited amount of unrelated sequence in place of most of the PH domain and the Arf GAP and ankyrin repeat domains (Fig. 4). The sequence of PIKE-S includes an N-terminal extension containing three SH3 domain-binding motifs also found in the splice variant of AGAP2 referred to as PIKE-L (Ahn and Ye, 2005; Ahn *et al.*, 2004a,b; Liu *et al.*, 2007). PIKE-S is found in the brain and localizes exclusively to the nucleus, where it appears to function in mitogenic and antiapoptotic signaling.

Because of the homology of PIKE-S to Ras, function analogous to Ras was investigated. PIKE-S was found to bind GTP or ATP with high affinity (10–40 nM) and a 1:1 stoichiometry (Ye *et al.*, 2000). GTP binding to PIKE-S in cells was increased by addition of NGF. PIKE-S bound to PI 3-kinase, a known target of Ras, dependent on GTP. The mode of interaction with PI 3-kinase, however, was more complex than described for Ras. PIKE-S-GTP simultaneously bound both the p85 catalytic and the p110 regulatory subunits of PI 3-kinase. The proline-rich domains of PIKE-S mediated binding to p110 and the Ras-like domain mediated binding to the catalytic p85 subunit. The association with both subunits was required to increase PI 3-kinase activity, leading to increased PIP3 production and activation of the antiapoptotic protein kinase Akt/PKB.

The signaling pathway leading from the NGF receptor to PIKE-S in the nucleus was further defined with the identification of PLC γ 1 as a PIKE-S-binding partner (Ye *et al.*, 2002). PLC γ 1 was found to bind to the third proline-rich domain (SH3-binding motif) of PIKE-S, via the SH3 domain of PLC γ 1. The association resulted in accelerated nucleotide exchange on PIKE-S; thus, PLC γ 1 functions as a GEF. Snyder and colleagues proposed a model in which NGF triggers PLC γ 1 translocation to the nucleus (Ye *et al.*, 2002). PLC γ 1 activates PIKE-S, which activates PI3-kinase, leading to the production of PIP3 and activation of the antiapoptotic kinase Akt/PKB.

PIKE-S is not found in humans, but humans do have two splice variants containing Arf GAP domains. Proteins with the Ras-like domain of PIKE-S, PH, Arf GAP, and ankyrin repeat domains, called AGAP2/GGAP2, were reported by Nie *et al.* (2002) and Liu and colleagues (Xia *et al.*, 2003). AGAP2 has also been called PIKE-A (Ahn *et al.*, 2004a,b). A third splice variant, PIKE-L, has the N-terminal proline-rich motifs found in PIKE-S in addition to the GTP-binding protein like PH, Arf GAP, and ankyrin repeat domains (Ahn *et al.*, 2004a; Liu *et al.*, 2007; Ye and Snyder, 2004). AGAP2 is a member of the AGAP subfamily of Arf GAPs (Inoue and Randazzo, 2007; Kahn *et al.*, 2008). The family is composed of 11 genes. AGAP1 and AGAP2 function as Arf1 GAPs that are activated by phosphoinositides (Nie *et al.*, 2002). In contrast to PIKE-S, AGAP1 and AGAP2 localized to an endocytic compartment (Nie *et al.*, 2003a, 2005). The PH domains of AGAP1 and AGAP2 bind to clathrin adaptor proteins, which are proteins important for membrane traffic. AGAP1 specifically binds clathrin adaptor AP-3 and regulates endocytic traffic from the TGN to lysosomes. AGAP2 specifically binds to AP-1 and affects transferrin recycling. Based on these results, AGAPs were proposed to function as a component of clathrin coats, which regulates membrane traffic.

The Ras-like domain of AGAP1/GGAP1 and AGAP2/GGAP2 has been reported to function like Ras (Xia *et al.*, 2003). In experiments using IP-purified protein, AGAP1, similar to PIKE-S, bound GTP with high affinity and hydrolyzed GTP at about 0.01/min. The $t_{1/2}$ for GDP dissociation was about 10 min. The GAP domain of the protein, through an intramolecular interaction, bound to the Ras-like domain and stimulated GTPase activity by about tenfold. As reported for PIKE-S, AGAP1/GGAP1 was found to stimulate the Ras pathway. In these experiments, activation of the c-fos serum response element was used as a reporter for the Ras pathway.

Further examination of signaling through AGAP2/PIKE-A and PIKE-L revealed differences from PIKE-S (Ahn and Ye, 2005; Ahn *et al.*, 2004a,b; Chan and Ye, 2007; Liu *et al.*, 2007; Ye, 2006). As described above, PIKE-S is thought to activate Akt consequent to binding to and activating PI 3-kinase. In contrast, PIKE-A/AGAP2 did not bind to PI 3-kinase. Instead, PIKE-A bound to and directly activated Akt, bypassing the need for PI 3-kinase activation. PIKE-L could also bind directly to Akt. Also different than the interaction of PIKE-S with PI 3-kinase, GTP binding to AGAP2/PIKE-A does not appear to regulate association with Akt (Ahn *et al.*, 2004a). AGAP2/PIKE-A binding to Akt was found to depend on either GTP or GDP (Ahn *et al.*, 2004b). Paradoxically, mutants of PIKE that would be predicted to reduce binding affinity for guanine nucleotide increased the binding of PIKE to Akt.

In summary, PIKE-S, PIKE-L, and PIKE-A/AGAP2 were all found to be important activators of Akt. For PIKE-S, the activation is dependent on GTP binding and is mediated by activation of PI 3-kinase. For PIKE-A, the activation is independent of the nucleotide bound and is mediated by direct binding to Akt.

AGAP2/PIKE-A has been linked to oncogenesis. AGAP2/PIKE-A, but not PIKE-L or PIKE-S, was found amplified in a number of human glioblastoma cell lines. Overexpression of AGAP2/PIKE-A increased Akt activity and promoted invasion of two glioblastoma cell lines (Ahn *et al.*, 2004b). In other studies, 154 tumors and corresponding normal tissues were examined (Ahn *et al.*, 2004a). PIKE-A/AGAP2 cDNA was elevated in multiple cancers, including breast, lung, ovary, kidney, bladder, vulva, uterus, and cervical. In NIH 3T3 fibroblasts and glioblastoma cell lines, expression of AGAP2/PIKE-A enhanced Akt activity, accelerated cell growth, and induced anchorage independent growth. The data support the idea that AGAP2 is oncogenic due to binding and activating Akt.

The evidence that AGAP2/PIKE-A has a role in oncogenesis is compelling; however, details of the mechanism remain to be determined. The literature contains conflicting reports about properties of AGAP2 important to its function as a proto-oncogene. For instance, Ye and colleagues (Hu *et al.*, 2005) reported robust and specific binding of PIP3 to PIKE-A/AGAP2 that was critical to the function of the protein. Nie *et al.* (2002) determined affinities of AGAPs for phosphoinositides. The proteins bound phosphoinositides without specificity for the position of the phosphate group on the inositol ring. Furthermore, the dissociation constant for PIP3 was greater than 10 μ M, which is considered low-affinity binding. The nucleotide-binding properties of AGAP2 and the ability to function as Ras are also controversial. Snyder and colleagues (Ye *et al.*, 2000) and Liu and colleagues (Xia *et al.*, 2003) reported that the AGAP family members bind GTP with high affinity, approximating the dissociation constant to be in the 10 nM range, and to hydrolyze GTP at a rate of ~0.01/min, similar to Ras. However, Nie *et al.* (2002) could not detect binding with 1 μ M or less nucleotide. On the basis of saturation kinetics carried out with highly purified AGAP, Declan and colleagues estimated the dissociation constant to be 500 μ M and the catalytic rate of GTP hydrolysis to be 10/min (Soundararajan *et al.*, 2007), each about a 1000-fold different than the parameter estimates provided by Liu and colleagues (Xia *et al.*, 2003) and Snyder and colleagues (Ye *et al.*, 2000). With a dissociation constant of 500 μ M, it is hard to understand how an effect of PLC on exchange rates could have been detected on the time scales of the experiments reported (Ye *et al.*, 2002). Snyder and colleagues and Liu and

colleagues report that the Ras-like domain of PIKE and other AGAPs activated Ras effectors (Ahn and Ye, 2005; Xia *et al.*, 2003; Ye and Snyder, 2004; Ye *et al.*, 2000); Nie and colleagues were not able to detect an effect of AGAP on a number of Ras effectors (Nie *et al.*, 2002). Finally, Liu and colleagues report that the GAP domain functions as a GAP for the Ras-like domain in an intramolecular reaction (Xia *et al.*, 2003). In contrast, Nie *et al.* (2002, 2003a, 2005) report that the GAP domain functions with Arf1·GTP.

B. ASAPs and Cell Invasion

Two ASAP subtype proteins (Fig. 5) have been implicated in carcinogenesis. They are thought to contribute to the invasive and metastatic phenotype by regulating cellular adhesive structures and the associated actin cytoskeleton.

1. ASAP1—ASAP1 was identified on the basis of Arf GAP activity and, separately, in screens for Src-binding proteins (Brown *et al.*, 1998; King *et al.*, 1999). ASAP1 was found to bind to and be phosphorylated by Src family proteins and focal adhesion kinase (FAK) and to associate with FAs (Brown *et al.*, 1998; Liu *et al.*, 2002, 2005; Randazzo *et al.*, 2000b). Studies in uveal melanoma were the first to reveal a role of ASAP1 in cancer (Ehlers *et al.*, 2005). The gene encoding ASAP1 is on chromosome 8q24.1, near the locus for the *myc* oncogene. The region of chromosome 8 containing ASAP1 is amplified in approximately half of uveal melanomas. These are class 2 tumors that have high invasive potential. After failing to detect changes in expression of *myc*, Harbour and colleagues (Ehlers *et al.*, 2005) found that ASAP1 message and proteins levels correlated with invasive potential in the class 2 uveal melanomas. Expressing recombinant ASAP1 increased the migration rate of cells derived from class 1 (noninvasive) uveal melanomas. Additional studies on the role of ASAP1 in the invasive behavior of uveal melanoma have been slowed because of difficulty in culturing cells from class 2 tumors.

The role of ASAP1 in invasion was also examined in breast and prostate cancer. ASAP1 levels correlated with invasive potential in primary tumors (Lin *et al.*, 2008; Onodera *et al.*, 2005). Similar to uveal melanoma, copy number of the ASAP1 gene was increased in primary prostate cancer specimens (Lin *et al.*, 2008), although this has not been reported for breast cancer. Different than the case for uveal melanoma, mammary carcinoma and prostate carcinoma cell lines could be established from the invasive tumors and were also found to contain high expression levels of ASAP1 (Onodera *et al.*, 2005; Sabe *et al.*, 2006). Consistent with a function in invasion, ASAP1 was found in and was required for the formation of invadopodia in mammary carcinoma cell lines (Bharti *et al.*, 2007; Onodera *et al.*, 2005) and in analogous structures called podosomes in Src-transformed fibroblasts (Bharti *et al.*, 2007).

Examination of colorectal carcinoma revealed that the potential function of ASAP1 in uveal melanoma and prostate cancer may not extend to all tumor types. Like uveal melanoma, amplification of chromosome 8q24.1 is frequently observed in colorectal carcinoma. However, in this case, amplification of the *myc* gene correlated with more advanced stages of cancer, whereas changes in the ASAP1 gene did not (Buffart *et al.*, 2005).

ASAP1 affects cell migration through association with cellular adhesive structures. Initial studies focused on the function of ASAP1 in FAs. Two proteins contribute to ASAP1 targeting to FAs. ASAP1 is a binding partner of FAK. The interaction is mediated by the SH3 domain of ASAP1 and is necessary for ASAP1 targeting to FAs in rat embryo fibroblasts (Liu *et al.*, 2002). Crk/CrkL, an adaptor protein, mediated ASAP1 association with FAs in platelets (Oda *et al.*, 2003). It binds to the proline-rich SH3-binding motifs present between the Ank repeat and E/DLPPKP repeat domains. ASAP1 overexpression was found to affect the paxillin content, but not vinculin content, of FAs (Liu *et al.*, 2002, 2005). The effect was dependent on Arf GAP activity. Reduced ASAP1 expression also reduced the association of paxillin with FAs and increased levels of Arf1·GTP, which led to the conclusion that the rate of the GTP binding and hydrolysis cycle on Arf1 regulated the association of paxillin, and possibly other proteins, with FAs (Liu *et al.*, 2005).

The effects of ASAP1 on cell movement are complex. Overexpressing ASAP1 has been reported to accelerate (Furman *et al.*, 2002) and to reduce cell migration rates (Liu *et al.*, 2005). Reduction of ASAP1 levels also reduced migration of fibroblasts (Furman *et al.*, 2002; Liu *et al.*, 2002, 2005). Reduction of ASAP1 has been reported to slow migration of breast and prostate cancer cells (Lin *et al.*, 2008; Onodera *et al.*, 2005) and, from another laboratory, to have no effect on the migration of cultured breast cancer cells (Ha *et al.*, 2008). Thus, the role of ASAP1 in cell migration may be specific to particular cell types and experimental conditions.

The biochemistry related to the function of ASAP1 in invadopodia and podosomes has been examined in several reports. One unresolved issue is related to ASAP1 splice variants and cortactin. ASAP1 has two major splice variants. ASAP1a contains three proline-rich SH3-binding motifs between the ankyrin repeats and the tandem repeats of E/DLPPKP. The middle motif is atypical, composed of a string of six prolines. The other splice variant, ASAP1b, does not contain the middle, atypical proline-rich SH3-binding motif. It has been reported that the middle motif, and consequently ASAP1a but not ASAP1b, binds to cortactin (Onodera *et al.*, 2005). The ASAP1a–cortactin complex drives formation of invadopodia. Perturbation of the interaction with a peptide composed of the atypical SH3-binding motif disrupted invadopodia formation (Hashimoto *et al.*, 2006; Onodera *et al.*, 2005). In contrast, another group reports that both ASAP1a and ASAP1b bound to cortactin and supported invadopodia and podosome formation. A peptide composed of the atypical proline-rich SH3-binding motif neither bound cortactin nor affected the formation of podosomes (Bharti *et al.*, 2007). The role of an atypical proline motif (PXXXPR) between the E/DLPPKP repeat and SH3 domains, which binds to Cin85, is also unclear. It was reported to be critical for the formation of invadopodia (Nam *et al.*, 2007). However, it did not contribute to the formation of podosomes (Bharti *et al.*, 2007). Some of the difference between groups could be attributed to cell type differences or the differences between podosomes and invadopodia.

The contribution of three other structures within ASAP1 to podosome formation has been examined (Bharti *et al.*, 2007). The tyrosine that is phosphorylated by Src was found to be critical to podosome formation. ASAP1 with the tyrosine mutated to phenylalanine functioned as a dominant negative, blocking podosome formation. ASAP1 with a glutamate

in place of the tyrosine supported podosome formation, but was not sufficient to drive podosome formation. The SH3 domain of ASAP1, which binds to FAK and is necessary for binding to cortactin, was also found to be critical to podosome formation. Recombinant ASAP1 with the SH3 deleted or containing point mutations within the SH3 domain functioned as a dominant negative, blocking formation of podosomes in fibroblasts expressing activated Src. The BAR domain also has a role in podosome formation. Recombinant ASAP1 that did not have the BAR domain could not support podosome formation but did not function as a dominant negative protein.

The role of the Arf GAP domain for invadopodia formation has been examined (Bharti *et al.*, 2007, and unpublished observations). Two types of mutants in the Arf GAP domain have been generated. In one type, Arf GAP activity is reduced or absent, but the mutant can still bind to Arf1·GTP. A second type of mutant is unable to bind Arf1·GTP. Mutants that can bind to Arf1·GTP support the formation of podosomes. In contrast, those mutant that cannot bind Arf1·GTP inhibit the formation of podosomes, functioning as dominant negative mutants. These results have been taken to support the idea that ASAP1 functions as an Arf effector.

Because ASAP1 is in invadopodia, it was anticipated to affect invasion. This hypothesis was tested in an invasive breast cancer cell line, MDA-MB-231. Reduction of ASAP1 expression, using siRNA, slowed invasion. Disrupting interaction with cortactin, using an SH3-binding peptide, slowed invasion as did disrupting the interaction of ASAP1 with CIN85 (Hashimoto *et al.*, 2006; Nam *et al.*, 2007; Onodera *et al.*, 2005). However, there may be some variation in the effect of ASAP1 in particular cell lines. Another group, also working with MDA-MB-231, did not detect an effect of reducing ASAP1 expression on invasion (Ha *et al.*, 2008).

In summary, ASAP1 contributes to the regulation of cellular adhesive structures involved in cell migration. The effect of ASAP1 in cancer invasion is cell type specific. The data are compelling that ASAP1 contributes to the invasive behavior in uveal melanoma. The role of ASAP1 in the behaviors of cancers of other origins is not well understood at this time.

2. ASAP3—ASAP3, previously called Upregulated in Liver Cancer 1 (UPLC1), DDEFL1, and ACAP4 (Fang *et al.*, 2006; Okabe *et al.*, 2004), was identified in analyses of expression profiles of clinical hepatocellular carcinomas (HCCs) using cDNA microarrays. ASAP3 is similar to ASAP1 and ASAP2 in structure but it does not contain an SH3 domain at the C-terminus (Fig. 5). Because it does not have an SH3 domain, the protein was called ACAP4 in one report. However, phylogenetic analysis indicates that the protein is an ASAP subtype Arf GAP and, therefore, has been renamed ASAP3 (Ha *et al.*, 2008; Kahn *et al.*, 2008) (Fig. 3A).

The identification of ASAP3 in expression profiles for HCCs led to the examination in cell lines derived from HCCs to determine a link to cancer. The relative expression of ASAP3 was reported to be high in HCC (Okabe *et al.*, 2004) and correlated with cell proliferation and migration (Fang *et al.*, 2006). These observations led to the hypotheses that high expression of ASAP3 causes increased cell proliferation and invasive behavior of cancer

cells. The effect of overexpressing ASAP3 on cell proliferation has not been consistent (Fang *et al.*, 2006; Ha *et al.*, 2008). Whereas one group found that expressing ASAP3 in NIH 3T3 fibroblasts increased proliferation, another group did not find any effect. Similarly, decreasing ASAP3 expression was found to slow proliferation in one report and to have no effect on proliferation in another report. The differences could be attributable to assay conditions or cell lines. Also, the positive effects observed were modest so it is possible that the assay used by one group lacked the sensitivity to detect changes in cell proliferation induced by ASAP3.

ASAP3 may control the interaction between cell adhesion structures and the actin cytoskeleton to regulate cell migration and invasion. ASAP3, like ASAP1, associates with FAs and circular dorsal ruffles, two structures involved in adhesion and cell migration (Buccione *et al.*, 2004). Unlike ASAP1, ASAP3 is not found in invadopodia. Also unlike ASAP1, overexpressing recombinant ASAP3 or reducing expression levels of ASAP3 did not affect the morphology of FAs, although a change in distribution of FAs was detected in some cell types. Reduction of ASAP3 expression decreased the number of actin stress fibers that bind to FAs and reduced the levels of phosphomyosin, which associates with and mediates the retraction of actin stress fibers (Ha *et al.*, 2008).

The effect of ASAP3 on movement of cells derived from a mammary carcinoma, MDA-MB-231, has been examined. Migration, assessed by a wound-healing assay and by a transwell migration assay, was slowed by reducing ASAP3 expression. Similarly, reduction of ASAP3 slowed migration through matrigel, taken as a measure of invasion. In the same experiments, reduction of ASAP1 had no effect (Ha *et al.*, 2008). The molecular bases for these effects are still being determined. Based on the available data, a model has been proposed in which ASAP3 controls the composition of FAs. Loss of ASAP3 results in the exclusion of actin-binding sites. Without the actin-binding sites, FAs cannot bind actin stress fibers, which prevents maturation of the FAs and prevents contraction that is necessary for cell movement.

In summary, ASAP3 affects migration and invasion of MDA-MB-231 cells, a mammary carcinoma cell line. ASAP3 does not have a large effect on cell proliferation and apoptosis has not been examined. The molecular basis for the effect on migration and invasion has not been explored.

VII. Arf GAP SUBTYPES THAT AFFECT SIGNALING OR ADHESION BUT HAVE NOT BEEN IMPLICATED IN ONCOGENESIS

A. The ARAPs

Three genes encode ARAP proteins in humans (Inoue and Randazzo, 2007; Kahn *et al.*, 2008). The ARAPs contain SAM, 5 PH, Arf GAP, ankyrin repeat, Rho GAP, and Ras association domains. The “R” stands for Rho GAP. Both the Arf GAP and Rho GAP domains contribute to the cellular effects of the ARAPs. Initial reports examined the effects of ARAP1 and ARAP3 on cell spreading and membrane ruffling (Krugmann *et al.*, 2002, 2004, 2006; Miura *et al.*, 2002; Stacey *et al.*, 2004). Recent work has provided some

molecular details about the mechanisms by which the ARAPs may affect cell movement. ARAP1 affects the endocytic traffic of EGFRs (Yoon, Lee and Randazzo, submitted) and ARAP2 affects the formation of FAs (Yoon *et al.*, 2006).

1. ARAP1—ARAP1 associates with the Golgi apparatus (Miura *et al.*, 2002) and, in cells treated with growth factors, a Rab5 pre-endosomal compartment involved in the endocytic traffic of EGFR (Yoon, Lee, and Randazzo, submitted). Reduced ARAP1 expression leads to more rapid internalization of EGFR following EGF treatment and reduced signaling through Erk and Jnk. On the basis of these results, the authors concluded that ARAP1 is involved in attenuation of signals from the EGFR but the molecular basis remains to be determined. Current studies are examining the role of the Rab5-containing pre-endosome in signaling.

2. ARAP2—ARAP2 is different from the other ARAPs in having a glutamine in place of the highly conserved catalytic arginine found in all known active Rho GAPs. The Rho GAP domain does not induce the hydrolysis of GTP bound to RhoA but it does bind specifically to RhoA-GTP. ARAP2 has PIP3-dependent Arf GAP activity that is specific for Arf6. ARAP2 is required for the formation of FAs. ARAP2 function to support FA formation depends on both the Arf GAP activity and the ability to bind to RhoA-GTP (Yoon *et al.*, 2006). These results are consistent with a role of ARAP2 in cell migration and/or invasion, but this function remains to be tested.

B. ACAPs and the Regulation of Integrin

ACAP proteins contain BAR, PH, Arf GAP, and ankyrin repeat domains (Inoue and Randazzo, 2007; Jackson *et al.*, 2000). *In vitro* and *in vivo* analyses indicate that ACAP1 and ACAP2 have a substrate preference for Arf6 over Arf1 and Arf5. ACAP1 is the most extensively studied of the ACAPs. Although not implicated in oncogenesis, ACAP1 does affect signaling pathways important to the behavior of cancer cells. It binds to phenylalanine-based sorting signals in transferrin receptor and promotes recycling of transferrin receptor (Dai *et al.*, 2004). ACAP1 also binds to integrin $\beta 1$ (Li *et al.*, 2005). The interaction is dependent on the phosphorylation of ACAP1 by Akt, and is regulated by EGFR. The interaction between ACAP1 and integrins regulates integrin recycling and controls cell migration.

VIII. COMPARATIVE ENZYMOLOGY OF THE Arf GAPs

PH domains distinguish the AZAP group from other Arf GAPs. The PH domains have been found to be critical for catalytic activity in ASAPs. Deletion of the PH domain of ASAP1 reduces activity to 1/100,000th that of wild-type protein (Che *et al.*, 2005; Kam *et al.*, 2000; Luo *et al.*, 2007). Similarly, deletion of the PH domain of ASAP2 and ASAP3 results in proteins with 1/1000th–1/10,000th the activity of the wild-type protein (Ha *et al.*, 2008). The role of the PH domain in other AZAP-type Arf GAPs has not been characterized. However, the role of phosphoinositides has been examined. The activity of Arf GAP1, which does not have a PH domain, is not affected by PIP2 or PIP3 (Randazzo, 1997). In contrast, ASAP1 is activated 10,000-fold by PIP2 (Che *et al.*, 2005; Kam *et al.*, 2000). ASAP2 and ASAP3 are similar to ASAP1 (Andreev *et al.*, 1999; Ha *et al.*, 2008). The Arf GAP activity of ARAPs is

specifically activated by PIP3 (Krugmann *et al.*, 2002; Miura *et al.*, 2002; Stacey *et al.*, 2004; Yoon *et al.*, 2006). AGAPs and ACAPs have been found to be activated by phosphoinositides but the effect is not specific for a particular phosphoinositide (Jackson *et al.*, 2000; Nie *et al.*, 2002).

The contribution of the PH domain for the GAP activity of AZAP proteins indicated that the AZAPs might differ catalytically from Arf GAPs that do not have PH domain. Consistent with this expectation, the amino terminus of Arf was found to be a critical determinant for interaction with ASAP1 and AGAP1 but not for Arf GAP1 (Yoon *et al.*, 2004). Studies focusing on structural determinants with the substrate Arf for interaction with GAP also revealed catalytic differences between AZAP subtypes (Luo *et al.*, 2005). A point mutation within switch 1 of Arf1, changing isoleucine 46 to aspartate, had a large effect on interaction with ASAP1 but not AGAP1 or Arf GAP1. These results were the basis for the conclusion that the GAPs, despite a similar catalytic fold, had distinct binding determinants for their substrates.

Examination of the enzymology of Arf GAP1 and ASAP1 revealed additional differences between the proteins (Luo and Randazzo, 2008; Luo *et al.*, 2007). The catalytic constants of ASAP1 and Arf GAP1 are more than 200-fold different. Structural studies indicate that the PH domain of ASAP1 forms part of the substrate-binding site, contributing both to binding and to orienting the substrate in the catalytic pocket. Arf GAP1 does not contain a PH domain (Luo and Randazzo, 2008). Differences also reside in the catalytic Arf GAP domain (Luo and Randazzo, 2008; Luo *et al.*, 2007). Mutation of tryptophan 479 within the Arf GAP domain of ASAP1 reduced activity to 1/4000th that of wild-type protein. The analogous mutation in Arf GAP1, tryptophan 32, reduced activity to one third that of wild-type protein, leading to the conclusion that this tryptophan is critical for ASAP1 but not Arf GAP1 catalytic activity.

IX. CONCLUSIONS

Thirty-one genes in humans encode Arf GAPs, proteins that induce the hydrolysis of GTP bound to the Arf family GTP-binding proteins. Of the 31 Arf GAPs, 4 (ASAP1, ASAP3, AGAP2, and SMAP1) have been found to be oncogenic. Five others (ARAP1, ARAP1, ACAP1, Git1, and Git2) have been found to affect signaling pathways that are often disrupted in cancer, but none of these has been directly implicated in oncogenesis. The effect of a particular Arf GAP may be specific for a tumor type. For instance, ASAP3, but not ASAP1, was found to affect the invasion of mammary tumor cells whereas ASAP1 contributes to the invasion of uveal melanoma and prostate cancer.

The molecular basis for Arf GAP action in cells is still being defined. Part of the action is through a common catalytic activity of inducing hydrolysis of GTP bound to Arf. Differences in function may arise from different sites of action or Arf isoform specificity. The Arf GAPs may also have other functions, either linked to or independent of Arf, as suggested by the structural diversity of these proteins. For instance, ARAPs, which have a Rho GAP domain, also exert effects by regulating Rho family GTP-binding proteins. If, as the early work indicates, AZAPs are critical mediators of cell migration related to metastasis

and invasion of specific cell types and the proteins can be molecularly distinguished, the Arf GAPs may be valuable therapeutic targets for the treatment of cancer.

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Fig. 1. Schematic representation of cellular adhesive structures regulated by Arf GAPs. (A) Focal adhesions (FA). The structures are illustrated in panels using ASAP3 which colocalizes with FA markers such as vinculin (left panels) and α -actinin (right panels) in U118 glioblastoma cells. (B) Invadopodia and podosomes. These structures are induced by Src activation. To visualize invadopodia and podosomes, cells were transfected with plasmids directing the expression of active Src. Under this condition, invadopodia and podosomes were detected using ASAP1 and cortactin in NIH3T3 fibroblasts (left panels, arrows indicate podosomes) and MDA-MB-231 breast cancer cells (right panels, arrows indicate invadopodia).

Fig. 2.

Model for Arf1 function in membrane traffic. Arf is activated by guanine nucleotide exchange factor (GEF) that catalyzes GTP exchange for GDP at the membrane. Next, the activated Arf-GTP recruits coat protein. The coat protein-Arf-GTP complex traps cargo (C). GTPase-activating protein (GAP) is then recruited to membrane sites through binding to the coat protein-Arf-GTP complex, triggering membrane deformation and subsequent vesicle formation. Fission releases the newly formed coated vesicle from the membrane. After vesicle budding, GAP hydrolyzes GTP on Arf, leading to the inactivation of Arf and subsequent dissociation of Arf and GAP from the cargo-containing vesicle. Coat proteins eventually dissociate from the vesicle surface, allowing the vesicle to dock and fuse with acceptor membranes.

Fig. 3.

Arf GAP family. (A) Arf GAP family phylogram. The phylogram, edited using Tree-View, was obtained from the multiple sequence alignment of Arf GAP domains using ClustalW2. The branch lengths in the phylogram are proportional to the estimated divergence along each branch. Accession #s for human Arf GAPs: Arf GAP1 = NM_018209; Arf GAP2 = NM_032389; Arf GAP3 = NM_014570; Git1 = NM_014030; Git2 = NM_057169; ASAP1 = NM_018482; ASAP2 = NM_003887; ASAP3 = NM_017707; ACAP1 = NM_014716; ACAP2 = NM_012287; ACAP3 = NM_030649; AGAP1 = NM_014914; AGAP2 = NM_014770; AGAP3 = NM_031946; AGAP4 = NM_133446; AGAP5 = XM_001132588; AGAP6 = NM_001077665; AGAP7 = NM_001077685; AGAP8 = NM_001077686; AGAP9 = XM_001716810; AGAP10 = XM_001714786; ARAP1 = NM_015242; ARAP2 = NM_015230; ARAP3 = NM_022481; SMAP1 = AY055004; SMAP2 = NM_022733. (B) Schematic of human Arf GAPs. All Arf GAPs have a conserved Arf GAP domain. The other domains are as follows: Ank, ankyrin repeats; BAR, Bin/Amphiphysin/Rvs; PBS, paxillin-binding site; PH, pleckstrin homology; SAM, sterile alpha motif; SH3, Src-homology 3; SHD, Spa2 homology domain; PRD, proline rich; GLD, GTP-binding domain.

Fig. 4.

Domain structures of three PIKE isoforms. PIKE-L, an alternatively spliced form of PIKE, is several hundred amino acids longer than the original form of PIKE, designated as PIKE-S. In addition to the GTP-binding domain (GLD) shared by PIKE-S and PIKE-L, PIKE-L contains an Arf GAP domain and two Ankyrin repeats. PIKE-S contains three proline-rich-domains (PRD) in the N-terminus, the GLD domain and a partial PH domain in the C-terminus. PIKE-A/AGAP2 contains the GTPase, PH, Arf GAP, and Ankyrin repeats domains present in PIKE-L but lacks the proline-rich domains (PRD) at the N terminus.

Fig. 5. Domain structures of the ASAP-type proteins of Arf GAPs. Amino acid sequence similarity between full-length ASAP1 and ASAP3 and each identified domain is indicated.

Table I

The Human AZAP-type Arf GAP Proteins and Their Alternative Names Found in the Literature

AZAPs	Accepted names	Alternative names
ASAP	ASAP1	DDEF1, AMAP1, PAG2, PAG β
	ASAP2	DDEF2, AMAP2, PAG3, PAP
	ASAP3	DDEFL1, UPLC1, ACAP4
AGAP	AGAP1	CENTG2, GGAP1
	AGAP2	CENTG1, GGAP2, PIKE-A
	AGAP3	CENTG3, CRAG, MRIP1
	AGAP4	CTGLF1, MRIP2
ARAP	ARAP1	CENTD2
	ARAP2	CENTD1, PARX
	ARAP3	CENTD3, DRAG1
ACAP	ACAP1	CENTB1
	ACAP2	CENTB2
	ACAP3	CENTB3