DEF-1/ASAP1 Is a GTPase-activating Protein (GAP) for ARF1 That Enhances Cell Motility through a GAP-dependent Mechanism*

Received for publication, September 21, 2001, and in revised form, December 20, 2001
Published, JBC Papers in Press, December 31, 2001, DOI 10.1074/jbc.M109149200

Craig Furman‡, Sarah M. Short‡, Romesh R. Subramanian‡, Bruce R. Zetter§, and Thomas M. Roberts††

From the ‡Department of Cancer Biology, Dana-Farber Cancer Institute, Harvard Medical School and the §Department of Surgical Research, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

DEF-1/ASAP1 is an ADP-ribosylation factor GTPase-activating protein (ARF GAP) that localizes to focal adhesions and is involved in cytoskeletal regulation. In this paper, we use a cell-based ARF GAP assay to demonstrate that DEF-1 functions as a GAP for ARF1 and not ARF6 in vivo. This degree of substrate preference was unique to DEF-1, as other ARF GAP proteins, ACAP1, ACAP2, and ARFGAP1, were able to function on both ARF1 and ARF6. Since transient overexpression of DEF-1 has been shown to interfere with focal adhesion formation and platelet-derived growth factor-induced membrane ruffling, we investigated whether NIH 3T3 cells stably expressing DEF-1 have altered cell motility. Here we report that ectopic DEF-1 enhances cell migration toward PDGF as well as IGF-1. This chemotactic effect appears to result from a general increase in cell motility, as DEF-1-expressing cells also exhibit enhanced levels of basal and chemokinetic motility. The increase in cell motility is dependent on DEF-1 GAP activity, since a DEF-1 mutant lacking the GAP domain failed to stimulate motility. This suggests that DEF-1 alters cell motility through the deactivation of ARF1. In contrast, the inhibition of cell spreading by DEF-1 was not dependent on GAP activity, indicating that spreading and motility are altered by DEF-1 through different pathways.

ADP-ribosylation factors (ARFs) represent a family of small G proteins that are involved in the regulation of membrane trafficking and the actin cytoskeleton (for reviews, see Refs. 1 and 2). There are six different mammalian isoforms of ARF, which have been divided into three classes based on sequence similarities. Class 1 consists of ARF1, ARF2, and ARF3; Class 2 consists of ARF4 and ARF5; and Class 3 is represented by ARF6 (3). Of the ARF family, ARF1 and ARF6 are the most thoroughly characterized. ARF1 predominantly localizes to the internal membranes of the Golgi apparatus and is important for endoplasmic reticulum to Golgi transport (4, 5), intra-Golgi transport (6, 7), endosome transport, and synaptic-vesicle formation (8, 9). ARF1 has also been implicated in the regulation of focal adhesions (FAs) (10). ARF6 localizes to the plasma membrane and endosomes, where it is thought to function in endocytosis (11, 12) and actin cytoskeleton organization (13), including the formation of membrane ruffles, filopodia, and actin-rich protrusions.

As with other G proteins, the activation state of ARFs is regulated by guanine nucleotides. ARFs are considered inactive in their GDP bound state and active when binding GTP. This cycle between GDP- and GTP-bound states is under the control of two groups of regulatory factors. The regulators that activate ARFs are GTPase exchange factors, which facilitate ARFs to release bound GDP, thus allowing for interaction with GTP. Deactivation is accomplished through the hydrolysis of bound GTP to GDP by the intrinsic GTPase activity of ARFs. Due to extremely low rates of intrinsic GTPase activity (14), ARFs require ARF GAPs, which stimulate their GTPase activity. The first ARF GAP to be described was ARFGAP1, a protein with a CXXC16CXXC zinc finger motif, which has proved to be the hallmark of the catalytically active ARF-GAP domain (15).

Another GAP protein with this zinc finger is DEF1/ASAP1 (differentiation enhancing factor-1/ARF GAP containing SH3, ANK repeat, and pleckstrin homology domains). DEF-1 was independently cloned based on its enzymatic ARF GAP activity as well as through its interaction with Src (16, 17). DEF-1 belongs to a family of related ARF GAP proteins, which consist of DEF-2/PAP, ACAP1, and ACAP2. In vitro ARF GAP assays have determined that DEF1 and DEF2 have a substrate preference for ARF1 and ARF5, with only modest activity toward ARF6 (16, 18). On the other hand, ACAP1 and ACAP2 possess greater activity toward ARF6 than to either ARF1 or ARF5 (19).

DEF1, DEF2, ACAP1, and ACAP2 are all involved in the regulation of the actin cytoskeleton (19–21). DEF-1 localizes to peripheral FAs, and overexpression of DEF-1 reduces the formation of FAs and PDGF-stimulated dorsal ruffles (20). These observations are consistent with DEF-1 functioning as a regulator of cytoskeletal remodeling.

Cell motility is a complex process that depends on the ability of a cell to form new FAs at the leading edge and to dissolve old FAs at its trailing edge (22, 23). This coordinated FA turnover provides the attachment points necessary for the cytoskeleton to pull the cell body in a given direction. Thus, disruption of FA turnover by DEF-1 expression would be predicted to alter the ability of cells to move. We have tested this prediction using a modified Boyden chamber cell motility assay and found that NIH 3T3 cells stably expressing DEF-1 have increased basal, chemokinetic, and chemotactic motility. These effects are me-
diated through DEF-1’s GAP activity, as a DEF-1 mutant lacking the GAP domain does not increase cell motility.

MATERIALS AND METHODS

Cell Culture—COS-1 (African green monkey kidney), and HEK 293 (human embryonic kidney) cells were grown in 10-cm diameter or six well tissue culture plates (Falcon) in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (JRH Biosciences), 100 units/ml penicillin G, and 100 μg/ml streptomycin-calcium (Invitrogen). NIH 3T3 cells were grown in 10% calf serum. All cells were grown at 37 °C in the presence of 7.5% CO2. HEK 293 transfections were performed using FuGENE 6 in accordance with the manufacturer’s instructions (Roche Molecular Biochemicals).

Antibodies—Full-length glutathione S-transferase-tagged DEF-1 protein was produced in the FastBac baculovirus/Sf21 system (Invitrogen), purified on glutathione-Sepharose beads (Amersham Biosciences, Inc.) and sent to Covance for the generation of an anti-DEF-1 polyclonal antibody. The resulting antiserum was purified by ImmunoPure (Aureus) IgG purification kit (Pierce). Anti-FLAG antibody (M5) was purchased from Sigma.

Mammalian Expression Constructs—Carboxy-terminal HA-tagged ARF1 and ARF6 cDNAs were subcloned from a pXS vector (11) into pcDNA3.1 (Invitrogen) using EcoRI and XhoI sites for ARF1 and EcoRI and XhoI sites for ARF6. DEF-1 expression vectors were constructed by ligation of full-length DEF-1 cDNA with an NH2-terminal FLAG-tag into a pcDNA3.1-DEF-1 ZFA was constructed by an in-frame deletion of amino acids 457–668 through the use of the endonuclease Ascll (New England Biolabs). FLAG-tagged DEF-1 and ZFA were also cloned into EcoRI and XhoI sites of the retroviral expression construct pWZL, which was modified by the inclusion of a cytomegalovirus promoter and EBNA ori.* Enhanced green fluorescent protein was cloned into HindIII and NotI sites of pcDNA3 and the BamHI site of pWZL. All constructs were verified by sequencing. The expression constructs for ARFGAP1, ACAP1, and ACAP2 have been described previously (19, 24).

Western Blots—Cells were washed in PBS and lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris (pH 8), 137 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.1 TIU of aprotinin/ml, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.75 μg/ml leupeptin). Cells were scraped, cleared at 13,000 × g and normalized for total protein levels using the Bradford assay (Bio-Rad). Lysates were mixed with 3× electrophoresis sample buffer, boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue and scanned for protein bands. The bands were excised, separated by in-gel digestion with trypsin (Sigma), and analyzed with a MALDI-TOF mass spectrometer. The tryptic digestions were analyzed with the Mascot search engine.

Immunofluorescence—NIH 3T3 cells stably expressing FLAG-tagged ARF1, ACAP1, and ACAP2 were ascertained by Western blot analysis. The expression constructs for ARFGAP1, ACAP1, or pCDNA3 vector. At 24 h post-transfection, cells were labeled with 1 μCi/ml [32P]orthophosphate (PerkinElmer Life Sciences) for 1 h at 37 °C. Cells were washed twice with 1× PBS, 5 min each and fixed for 5 min in 2% paraformaldehyde/PBS at room temperature. Permeabilization was achieved in methanol (2 min, −20 °C), followed by two 1× PBS washes and further permeabilized in 0.2% Triton X-100, PBS for 5 min at room temperature. Non-specific signal was eliminated by incubating cells 30 min with 1% BSA/PBS. Primary antibodies diluted 1:400 in 1% BSA/PBS were applied to cells for 1 h at RT (anti-axin, P13520, BD Transduction Laboratories, Lexington, KY; anti-FAK, 05–537, Upstate Biotechnology, Inc., Lake Placid, NY; M5 anti-Flag, F4042, Sigma-Aldrich). Cy3-labeled goat anti-mouse IgG (115–165–003, Jackson ImmunoResearch, West Grove, PA) was used as secondary antibody at a dilution of 1:1000 and was applied to cells for 1 h at room temperature. Confocal microscopy (Zeiss LSM410) was used to detect intracellular protein localization.

RESULTS

Mutating the DEF-1 ARF GAP Domain—Sequence comparison shows that all mammalian ARF GAPs have a zinc finger GAP domain similar to that of ARFGAP1. Fig. 1 compares the structural architecture of DEF-1 and related ARF GAP proteins with ARFGAP1. DEF-2 is most closely related to DEF-1 with identical domain organization except for a lack of the GDLPPKP repetitive motif found in DEF-1, ACAP1 and ACAP2 both terminate after the ankyrin repeats and thus lack...
the putative SH3 binding sites as well as the COOH-terminal SH3 domain (19). As noted by Brown et al. (16), this subset of GAP proteins have the core catalytic zinc finger flanked by an NH₂-terminal pleckstrin homology domain and COOH-terminal ANK repeats. This sequence of pleckstrin homology-zinc finger-ANK motifs has been termed the PZA module (16). While the zinc finger is sufficient for GAP activity in the case of ARFGAP1 (15), PZA containing ARF GAPs require the entire module for GAP activity (16, 18, 27). The crystal structure of this region of DEF-2 demonstrates that the first two ankyrin repeats form an extensive interface with the zinc finger domain and generates a stable structure (27). To create a GAP-inactive DEF-1, we deleted amino acids 457–668, corresponding to the zinc finger and first two ankyrin repeats, as shown in Fig. 1 and termed the deleted DEF-1 protein ∆ZA. This deletion was designed to eliminate GAP activity without disrupting conformation or stability. Furthermore, as this region also forms interactions with the ARF substrate, ∆ZA is unlikely to form an unproductive complex with ARF and thus act in an interfering manner. Western blot analysis shows that ∆ZA is expressed at levels comparable with DEF-1 (Fig. 2B).

DEF-1 Is an ARF-GAP in Whole Cells—To determine the substrate specificity of DEF-1 in the context of whole cells and to ensure that ∆ZA lacks GAP activity, the effect of expression of these proteins on the nucleotide binding state of ARFs was measured in intact cells. We investigated activity toward ARF1 and ARF6, the two most extensively characterized ARF family members. Cells co-expressing HA-tagged ARF1 or ARF6 and DEF-1 proteins were labeled with [32P]orthophosphate and lysed. Ectopic ARFs were immunoprecipitated from cell lysates and their activation state determined by quantitating the amount of radiolabeled GTP or GDP that co-precipitated with ARF after resolution of GTP and GDP by thin layer chromatography.

To validate the cell-based GAP assay we first determined the nucleotide binding state of wild type ARF as compared with the binding state of active and inactive mutants of ARF. The basal activation states of ARF1 and ARF6, as measured in the absence of an overexpressed ARF GAP, revealed that roughly twice as much ARF6 was in its active, GTP-bound state as compared with ARF1 (14.64 ± 1.5% versus 8.2 ± 0.73%; Student’s t test; p = 0.001). As expected, the inactive mutants ARF1 T31N and ARF6 T27N failed to exhibit detectable levels of GTP binding, while 56% of the active point mutant ARF1 Q71L was GTP-bound (Fig. 2A).

We then measured the effect of DEF-1 expression on ARF activation. The relative expression levels of ARFs and DEF-1 proteins under the conditions of the ARF GAP assay were determined by Western blot analysis (Fig. 2B). Fig. 2C shows a representative chromatographic separation from one experiment in which expression of DEF-1 reduced the amount of GTP bound by ARF1 from 10.3 to 5.1% but did not cause any decrease in ARF6 activation. Results from several experiments were expressed as the percentage change in ARF activation as compared with basal ARF activation (Fig. 2, D and E). DEF-1 exhibited GAP activity when using ARF1 as substrate, reducing the basal activation state of ARF1 by 50% (Fig. 2D). This activity is specific to the GAP domain of DEF-1, since ∆ZA, which has a deletion of the GAP domain, was enzymatically inactive. In contrast to its activity toward ARF1, DEF-1 demonstrated no GAP activity toward ARF6 as substrate (Fig. 2E).

In fact, while DEF-1 reduced the basal activation state of ARF1 by roughly 50%, it increased the activation state of ARF6 by 15%. While this slight activation was reproduced in three separate experiments, it is important to point out that it just fails to achieve the 95th confidence interval of statistical significance (paired Student’s t test; p = 0.067). To compare DEF-1’s substrate preference to other ARF GAPs, we also tested ACAP1, ACAP2, and ARF GAP1 for GAP activity. Unlike DEF-1, these other GAPs could utilize both ARF1 and ARF6 as substrate (Fig. 2, D and E).

DEF-1 Enhances Migration toward PDGF—After having demonstrated that DEF-1 functions as a GAP for ARF1 in intact cells, and that ∆ZA is, as expected, deficient in GAP activity, we looked for GAP-dependent effects of DEF-1 on the cytoskeleton.

Given the importance of FA turnover on cell motility and the report that DEF-1 overexpression destabilizes FAs, DEF-1 was tested for effects on cell motility. While previous DEF-1 cytoskeletal studies were performed under transient transfection conditions with high levels of overexpression, we studied the effect of lower levels of DEF-1 expression using retrovirally generated pooled populations of NIH 3T3 cells stably expressing DEF-1 or ∆ZA. Expression levels of ectopic DEF-1 proteins were determined to be ~10-fold over endogenous as determined by Western blotting (Fig. 3A). These stable pooled populations were assessed for chemotactic migration toward PDGF, a known chemoattractant for fibroblasts. To determine the optimal concentration of PDGF, a dose-response curve was performed using control NIH 3T3 pooled population (Fig. 3B). Cells were plated on porous, fibronectin-coated transwells suspended above a chamber containing DMEM or DMEM supplemented with 0, 1, 5, or 25 ng/ml PDGF. Migration was assessed by counting the number of cells that moved through the pores of the transwell toward the chemoattractant during a 4-h period. The number of cells migrating through the transwells increased with greater concentrations of PDGF up to the highest tested concentration of 25 ng/ml. Subsequent studies on the effect of DEF-1 or ∆ZA expression on cell migration were then measured using a subsaturating concentration of 20 ng/ml PDGF. Notably, DEF-1 stable cells exhibited an enhanced chemotactic migration response to PDGF, resulting in a 55% increase in the number of cells migrating to the chemoattractant over a 4-h period (Fig. 3C). The reproducibility of this enhancement was demonstrated in the course of several experiments using independently generated NIH 3T3 pooled populations. DEF-1 motility enhancement was dependent on GAP activity,
as /H9004 ZA behaved similar to the control. DEF-1 stable cell lines also exhibited a slight increase in basal motility as seen by migration in the absence of PDGF. To further investigate this enhancement of non-directed migration by DEF-1, a checkerboard migration assay was performed in which increasing concentrations of PDGF were added to the top and/or bottom chambers of the transwell (Fig. 4). Consistent with results in Fig. 3C, NIH 3T3 cells stably expressing DEF-1 exhibited enhanced basal migration, as determined in the transwells without PDGF in either top or bottom chambers. Conditions with increasing concentrations of PDGF in only the bottom chamber show that DEF-1 enhances chemotaxis in a dose-responsive manner. Conditions with equal concentrations of PDGF in both top and bottom chambers reveal that PDGF is capable of stimulating cell motility in the absence of a gradient (chemokinesis) and that DEF-1 expression enhances this chemokinetic response.

These findings suggest that enhanced chemotactic response of DEF-1 expressing NIH 3T3 cells, rather than being specific to PDGF, appears to be the result of a general enhancement of cell motility. Consistent with this model, DEF-1 stable NIH 3T3s were also found to exhibit increased motility when stimulated with another chemoattractant, IGF-1 (Fig. 5).

Expression of DEF-1 Interferes with Cell Spreading—As mentioned previously, cells need to orchestrate FA formation and dissolution to exhibit motility. Therefore, one possible explanation for DEF-1’s enhancement of cell motility is an increase in FA turnover, rendering the cell better able to form new FAs at its leading edge and dissolve FAs at its trailing edge. Such a disruption in FA formation by DEF-1 would be expected to also have an effect on cell spreading, and indeed, DEF-1 transfected NIH 3T3 cells have been shown to exhibit defects in cell spreading (20). To determine the effect of the GAP-inactive /H9004 ZA on cell spreading, and to compare it to DEF-1, two different systems were used. In a transient expression assay, HEK 293 cultures transfected with DEF-1 expression constructs were observed for changes in morphology. Cells overexpressing DEF-1 were less spread on tissue culture plates as compared with non-expressing control cells, with a subpopulation of these cells becoming extremely rounded (Fig. 6A). To quantitate this disruption of cell spreading, cultures of HEK 293 cells transfected with wild type DEF-1 or /H9004 ZA were scored as either spread or completely rounded. Intermediate phenotypes were counted as spread (Fig. 6B). While the majority of cells expressing DEF-1 were scored as spread by this criterion, roughly 10% were rounded up as compared with 2% for the vector control. This modest effect on cell spreading contrasts to that observed for /H9004 ZA expression, which caused ~50% of transfecants to become rounded. To determine expression levels of
DEF-1 proteins, lysates were prepared from the transfectants and subjected to Western blot analysis. Results from two independent transfections are shown in Fig. 6C. To determine whether this disruption of cell spreading could be observed at the lower expression levels attained in the migration assays, we employed the same DEF-1 stable NIH 3T3 pooled populations. These pooled populations were placed in suspension, replated on fibronectin-coated tissue culture plates and allowed to attach for 20, 40, or 120 min. Cells were then fixed and Coomassie stained. The degree of cell spreading was determined by measuring projected surface area of individual cells. The rate of cell spreading was delayed in cells expressing DEF-1 and, to a slightly greater degree, by /H9004ZA (Fig. 6D). This difference in the spreading rate resulted in an appreciable difference in projected surface area at the 40-min time period, when the control population had already achieved its maximum projected area but DEF-1 and /H9004ZA stable cell lines had not. By 120 min DEF-1 and /H9004ZA populations had largely caught up with the control.

To determine whether the effect of DEF-1 on spreading was attributable to a disruption of FAs, immunofluorescence microscopy was performed on DEF-1 and /H9004ZA stable NIH 3T3 pooled populations that had been allowed to fully attach to glass coverslips for 24 h. At this time point, ectopic expression of DEF-1 or /H9004ZA did not have an appreciable effect on FAK localization as compared with control NIH 3T3s (Fig. 7).

DEF-1 proteins, lysates were prepared from the transfectants and subjected to Western blot analysis. Results from two independent transfections are shown in Fig. 6C. To determine whether this disruption of cell spreading could be observed at the lower expression levels attained in the migration assays, we employed the same DEF-1 stable NIH 3T3 pooled populations. These pooled populations were placed in suspension, replated on fibronectin-coated tissue culture plates and allowed to attach for 20, 40, or 120 min. Cells were then fixed and Coomassie stained. The degree of cell spreading was determined by measuring projected surface area of individual cells. The rate of cell spreading was delayed in cells expressing DEF-1 and, to a slightly greater degree, by /H9004ZA (Fig. 6D). This difference in the spreading rate resulted in an appreciable difference in projected surface area at the 40-min time period, when the control population had already achieved its maximum projected area but DEF-1 and /H9004ZA stable cell lines had not. By 120 min DEF-1 and /H9004ZA populations had largely caught up with the control.

To determine whether the effect of DEF-1 on spreading was attributable to a disruption of FAs, immunofluorescence microscopy was performed on DEF-1 and /H9004ZA stable NIH 3T3 pooled populations that had been allowed to fully attach to glass coverslips for 24 h. At this time point, ectopic expression of DEF-1 or /H9004ZA did not have an appreciable effect on FAK localization as compared with control NIH 3T3s (Fig. 7). Similarly, paxillin staining appeared to be unperturbed in fully
attached cells expressing DEF-1. In contrast, expression of ΔZA had a strong influence on paxillin localization, resulting in a redistribution of paxillin from linear FAs to punctate structures in the cytoplasm and the perinuclear region.

Both transient and stable assays reveal that DEF-1 expression interferes with cell attachment to substrate. By decreasing cell adherence, DEF-1 could enhance cell motility, whether random, chemotactic, or chemokinetic. However, ΔZA, which interferes with cell attachment and paxillin localization to a greater degree than DEF-1, has no effect on cell motility. This suggests that the general stimulatory effect of DEF-1 on cell motility is not caused by a decrease in cell attachment.

**DISCUSSION**

DEF-1/ASAP1 and the related proteins, DEF2/PAP, ACAP1, and ACAP2, have all been shown to possess ARF GAP activity, thus functioning as negative regulators of ARFs. Each member of this family has also been implicated in the regulation of...
cytoskeleton, a process in which both ARF1 and ARF6 are known to play a role. In vitro GAP assays have revealed that this family of GAPs have varying degrees of substrate promiscuity for ARF1 and ARF6, making it difficult to ascertain their physiological substrates by this technique (16, 18, 19).

To compare the effect of DEF-1 and other ARF GAPs on ARF activation in a more physiological context, we employed a cell-based ARF GAP assay. There are several potential advantages to this approach over an in vitro GAP assay. First, the substrate preference of these GAPs could be more tightly regulated in the context of whole cells. For example, cells might normally restrict substrate access by maintaining GAPs and certain ARFs in non-overlapping subcellular compartments. Such an enhancement of specificity was not generally observed, as GAP proteins tended to show less substrate preference in the cell-based assay than in the in vitro assay. This does not rule out the possibility that distinct subcellular localization normally assists in substrate specificity, since the overexpression of GAP and ARF proteins necessary for the cell-based ARF GAP assay may have led to mislocalization. Interestingly, the one exception to this trend is DEF-1. Previous in vitro assays have shown that DEF-1 has a small but detectable activity toward ARF6, but such activity was undetected in the cell-based GAP assay. It is tempting to suggest that DEF-1 demonstrates greater substrate specificity than ARFGAP1, ACAP1, and ACAP2, because it is more highly regulated. The SH3 binding sites and SH3 domain, which are unique to DEF-1, might confer this specificity.

Not only did DEF-1 fail to function as a GAP on ARF6, it appeared to exhibit a slight stimulatory effect toward ARF6. Indeed, this observation may reveal another potential advantage of a cell-based GAP assay; the ability to detect indirect effects on ARF activation. There is considerable precedent for this type of indirect effect in the case of other small G proteins. For example, changes in the GTP loading state of p21Ras can alter the activation state of Rac or Rho (28). Furthermore, Rho family members can function as a hierarchical signaling cascade, with Cdc42 activating Rac, and Rac activating Rho (29–31) or, in certain cases, with Cdc42 acting antagonistically toward Rac and Rho (32, 33). If such interplay exists within the ARF family, deactivation of ARF1 by DEF-1 might lead to the activation of ARF6. However, it seems highly unlikely that ARF1 directly influences ARF6 activation, since other GAP proteins that inactivate ARF1 had no stimulatory effect on ARF6. Furthermore, ΔZA, which did not inactivate ARF1, still had a stimulatory effect on ARF6. These observations do not rule out the possibility that DEF-1 itself is part of a negative feedback mechanism involved in coordinating ARF1 and ARF6 activation states. Such a function could be accomplished if DEF-1 sequesters co-factors necessary for the normal regulation of ARF6.

A DEF-1 mutant lacking GAP activity was necessary to determine whether DEF-1 effects on motility and cell spreading were dependent on GAP activity. To generate a DEF-1 mutant that was GAP inert, and not acting in a dominant interfering manner, the zinc finger and first two ankyrin repeats were removed. As these motifs fold into a stable structure that is required for GAP activity, their deletion was expected to result in a DEF-1 protein lacking GAP activity. Furthermore, as this region is thought to form contacts with ARF, the resulting mutant should be unable to interact with ARF and thus form unproductive complexes which, by interfering with endogenous GAPs, could act as a dominant negative. Because DEF-1 mutants that form unproductive complexes would appear merely GAP-dead in an in vitro GAP assay, the cell-based approach had the advantage of enabling us to demonstrate that ΔZA did not act as a dominant negative with regard to ARF1 activity.

Several lines of evidence indicate that DEF-1 is involved in cytoskeletal regulation. DEF-1 localizes to FAs and interacts with FAK in yeast two-hybrid assays (20). Furthermore, transient overexpression of DEF-1 interferes with PDGF-induced FA turnover and dorsal ruffle formation, suggesting that DEF-1 is involved in PDGF-mediated signaling. Since FAs serve as contact points for the generation of mechanical forces required for cell spreading and movement, suppression of FA formation by DEF-1 expression raised the possibility that DEF-1 might affect PDGF-directed chemotaxis. Indeed, we found that DEF-1 expressing NIH 3T3 cells exhibited an increase in chemotaxis toward PDGF. This stimulatory effect was dependent on DEF-1 GAP activity, as cells expressing a DEF-1 mutant with the GAP domain deleted, ΔZA, behaved like control.

The enhancement of motility by DEF-1 did not appear to be specific to PDGF signaling, since we noticed that there was a consistent increase in basal migration of DEF-1 expressing NIH 3T3 cells in the absence of PDGF. This observation was more fully explored by a checkerboard migration assay, in which PDGF is added in various combinations to the top and bottom chambers of the transwells. The checkerboard assay thus exposes cells to different PDGF gradients or PDGF in the absence of a gradient. Results show that DEF-1 expression increases basal migration rates in the absence of PDGF as well as PDGF-stimulated chemokinesis. The fact that DEF-1 expression increases basal migration suggests that the increased chemotactic and chemokinetic response to PDGF is caused by a general predisposition of these DEF-1 expressing cells to move and not to an enhanced PDGF-specific response. Supporting this conclusion is the fact that DEF-1 expressing NIH 3T3 cells also exhibited enhanced migration toward another chemotactant, IGF-1. Finally, cell lysates were prepared from control and DEF-1 NIH 3T3 cells that had been stimulated with PDGF over a 4-h time course. These whole cell lysates, as well as PDGF receptor immunoprecipitated from the lysates, were subjected to a Western blot analysis using an anti-phosphotyrosine antibody. DEF-1 NIH 3T3 cells exhibited time-dependent PDGF receptor activation and a phosphorylation pattern of total lysates that was indistinguishable from control cells (data not shown). This suggests that DEF-1 expression does not influence signal transduction in response to PDGF.

A possible model for the general enhancement of cell motility by DEF-1 could depend on the ability of DEF-1 to destabilize FAs. Such a destabilization could lead to an increase in overall FA turnover, resulting in an increase in basal, chemokinetic, and chemotactic migration rates. FA disruption has already been documented in DEF-1 transiently transfected NIH 3T3 cells, but the level of overexpression in this case was higher than that obtained in our retroviral productions DEF NIH 3T3 stable pooled populations (25–50-fold in the transient system versus 10-fold for the stable populations). At this lower level of expression, DEF-1 did not grossly disrupt FAs as determined by immunofluorescence microscopy for FAK and paxillin. We did find, however, that even at this expression level ectopic DEF-1 could still interfere with cell adhesion, a process that is dependent on proper FA functioning. These observations indicate that while low level DEF-1 expression is insufficient to disrupt FAs, it may be capable of altering the turnover rate of FAs or affecting FA function in some other manner. Nevertheless, such a possible modification of FA function by DEF-1 cannot fully explain the enhancement of motility by DEF-1,

3 C. Furman and T. M. Roberts, unpublished results.
since ∆ZA, which does not stimulate cell motility, was found to be more potent than DEF-1 in its ability to interfere with cell spreading.

Intriguingly, expression of ∆ZA also caused a relocation of paxillin away from linear focal adhesions in a process that did not affect the presence of FAs, as determined by immunofluorescence using another DEF-1 GAP mutant suggests that under physiological conditions the recruitment of paxillin to FAs is regulated by DEF-1.

The observation that ∆ZA inhibits cell spreading to a greater degree than DEF-1 is in contrast with previously published results using another DEF-1 GAP mutant with a key arginine to lysine point mutation, R497K (20). While R497K did disrupt cell spreading, it did so less effectively than DEF-1. The reason for this discrepancy may depend on the different function of these two mutants. ∆ZA lacks GAP activity due to a large deletion that removes the enzymatic site as well as the region involved in interaction with substrate. The DEF-1 point mutant, R497K, is thought to lack GAP activity since the targeted residue provides the conserved arginine finger necessary to stabilize the ARF GTP/GDP transition state (34). This mutant still possesses several other conserved hydrophobic residues predicted to facilitate the interaction of DEF-1 with ARF1. Thus, expression of DEF-1 R497K may sequester ARF into non-productive complexes and actually function as a dominant negative, a possibility raised by Randazzo et al. (20).

The fact that the increase in cell motility correlates with DEF-1 GAP activity suggests this effect is mediated by the deactivation of the DEF-1 substrate, ARF1. The mechanism by which ARF1 influences motility is currently unknown, although ARFs are well established regulators of the cytoskeleton. There is evidence that ARFs may influence activity of the Rho family of G proteins, which regulate the cytoskeleton and cell motility (35).

The ability of DEF-1 to increase motility does not appear to result from a decrease in cell spreading. In fact, cell spreading does not appear to be mediated by ARF1 activity, since the GAP-inactive ∆ZA is still capable of interfering with spreading. Interestingly, the interference of spreading by DEF-1 and ∆ZA does correlate with our observation that both proteins may increase the activity of ARF6. While ARF6 is involved in the regulation of cell spreading, the potential physiological significance of ARF6 activation by DEF-1 is unknown. With its numerous domains not required for GAP activity, it is possible that the DEF-1 spreading effect is mediated by a non-GAP-dependent mechanism.

Recently, ARF6 activation has been demonstrated to increase cell motility (36, 37). Our finding that ARF1 also influences cell motility suggests that the ARF family of G proteins plays a general role in regulating cellular migration. Interestingly, ARF1 and ARF6 appear to function antagonistically, with ARF1 activation being antimigratory and ARF6 activation being promigratory. While the mechanism that links ARFs with the cellular migration machinery is poorly understood, it is clear that regulators of ARF activity, such as DEF-1, are important components of the overall system.

Acknowledgment—We thank Ole Gjoerup for helpful discussions and critical review of the manuscript.

REFERENCES

1. Chavrier, P., and Goud, B. (1999) *Curr. Opin. Cell Biol.* 11, 466–475
2. Randazzo, P. A., Nie, Z., Miura, K., and Hsu, V. W. (2000) *Science* 289, 1–15
3. Moss, J., and Vaughan, M. (1998) *J. Biol. Chem.* 273, 21431–21434
4. Dascher, C., and Balch, W. E. (1994) *J. Biol. Chem.* 269, 1437–1448
5. Zhang, C., Rosenwald, A. G., Wllingham, M. C., Skuntz, S., Clark, J., and Kahn, R. A. (1994) *J. Cell Biol.* 124, 289–300
6. Rosano, J. E. (1994) *J. Cell Biol.* 124, 1311–1317
7. Coisson, P. (1997) *Curr. Opin. Cell Biol.* 9, 484–487
8. Donaldson, J. G., and Klausner, R. D. (1994) *Curr. Opin. Cell Biol.* 6, 527–532
9. Fossel, V., Horn, J. T., and Kelly, R. B. (1998) *Cell* 93, 425–432
10. Norman, J. C., Jones, D., Barry, S. T., Holt, M. R., Cockcroft, S., and Critchley, D. R. (1998) *J. Cell Biol.* 143, 1861–1995
11. Peters, P. J., Hsu, V. W., Ost, C. E., Finazzi, D., Teal, S. R., Oorschot, V., Donaldson, J. G., and Klausner, R. D. (1995) *J. Cell Biol.* 128, 1003–1017
12. D’Souza-Schorey, C., van Dorselaer, E., Hsu, V. W., Yang, C., Stahl, P. D., and Peters, P. J. (1998) *J. Cell Biol.* 140, 663–666
13. Radhakrishna, H., Klausner, R. D., and Donaldson, J. G. (1996) *J. Cell Biol.* 134, 953–947
14. Kahn, R. A., and Gilman, A. G. (1986) *J. Biol. Chem.* 261, 7906–7911
15. Cukierman, E., Huber, I., Rotman, M., and Cassel, D. (1996) *Science* 270, 1999–2002
16. Brown, M. T., Andrade, J., Radhakrishna, H., Donaldson, J. G., Cooper, J. A., and Randazzo, P. A. (1998) *Mol. Cell. Biol.* 18, 7038–7051
17. King, P. J., Hu, E., Harris, D. F., Sarraf, P., Spiegelman, B. M., and Roberts, T. M. (1999) *Mol. Cell. Biol.* 19, 2330–2337
18. Andreev, J., Simon, J. P., Sabatini, D. D., Kam, J., Plowman, G., Randazzo, P. A., and Schlessinger, J. (1999) *Mol. Cell. Biol.* 19, 2358–2366
19. Jackson, T. R., Brown, F. D., Nie, Z., Miura, K., Foroni, L., Sun, J., Hsu, V. W., Donaldson, J. G., and Randazzo, P. A. (2000) *J. Cell Biol.* 151, 627–638
20. Randazzo, P. A., Andrade, J., Miura, K., Brown, M. T., Long, Y. Q., Stauffer, S., Roller, P., and Cooper, J. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 4011–4016
21. Kondo, A., Hashimoto, S., Yano, H., Nagayama, K., Maza, Y., and Sabe, H. (2000) *Mol. Cell.* 5, 129–137
22. Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988) *Annu. Rev. Cell Biol.* 4, 487–525
23. Stossel, T. P. (1993) *Cell* 74, 1086–1094
24. Iriti, H., Cukierman, E., Rotman, M., Ase, T., Hsu, V., and Cassel, D. (1998) *J. Biol. Chem.* 273, 24786–24791
25. Langille, S. E., Patki, V., Klarlund, K., Buxton, J. M., Holik, J. J., Chowla, A., Curvera, S., and Czech, M. P. (1999) *J. Biol. Chem.* 274, 27099–27104
26. Chen, C., and Okumura, H. (1987) *Mol. Cell. Biol.* 7, 2745–2752
27. Mandiyan, V., Andreev, J., Schlessinger, J., and Hubbard, S. R. (1999) *EMBO J.* 18, 6980–6988
28. Nimnuan, A. S., Yatsula, B. A., and Bar-Sagi, D. (1998) *Science* 279, 560–563
29. Ridley, A., and Hall, A. (1992) *Cell* 70, 389–399
30. Nobes, C. D., and Hall, A. (1995) *Cell* 81, 53–62
31. Peppelenbosch, M. P., Qiu, R. G., de Vries-Smits, A. M., Tertoolen, L. G., de Laat, S. W., McCormick, F., Hall, A., Symons, M. H., and Bos, J. L. (1995) *Cell* 81, 849–856
32. Kozma, R. A., Ahmed, S., Best, A., and Lim, D. (1995) *Mol. Cell. Biol.* 15, 1942–1952
33. Kozma, R., Sarner, S., Ahmed, S., and Lim, D. (1997) *Mol. Cell. Biol.* 17, 1201–1211
34. Scheffere, K., Ahmadian, M. R., and Wittinghofer, A. (1998) *Trends Biochem. Sci.* 23, 257–262
35. Hall, A. (1998) *Science* 279, 509–514
36. Santc, L. C., and Casanova, L. E. (2001) *J. Cell Biol.* 154, 599–610
37. Palacios, F., Price, L., Schweitzer, J., Collard, J. G., and D’Souza-Schorey, C. (2001) *EMBO J.* 20, 4973–4986
DEF-1/ASAP1 Is a GTPase-activating Protein (GAP) for ARF1 That Enhances Cell Motility through a GAP-dependent Mechanism
Craig Furman, Sarah M. Short, Romesh R. Subramanian, Bruce R. Zetter and Thomas M. Roberts

J. Biol. Chem. 2002, 277:7962-7969.
doi: 10.1074/jbc.M109149200 originally published online December 31, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109149200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 25 of which can be accessed free at http://www.jbc.org/content/277/10/7962.full.html#ref-list-1