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Report

The Arf6 GEF GEP100/BRAG2 Regulates Cell Adhesion by Controlling Endocytosis of β1 Integrins

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Summary

The small GTPase Arf6 has been shown to regulate the post-endocytic trafficking of a subset of membrane proteins, including β1 integrins, and inhibition of Arf6 function impairs both cell adhesion and motility [1]. The activity of Arf GTPases is regulated by a large family of guanine nucleotide exchange factors (GEFs) [2]. Arf-GEP100/BRAG2 is a GEF with reported specificity for Arf6 in vitro [3], but it is otherwise poorly characterized. Here we report that BRAG2 exists in two ubiquitously expressed isoforms, which we call BRAG2a and BRAG2b, both of which can activate Arf6 in vivo. Depletion of endogenous BRAG2 by siRNA leads to dramatic effects in the cell periphery; one such effect is an accumulation of β1 integrin on the cell surface and a corresponding enhancement of cell attachment and spreading on fibronectin-coated substrates. In contrast, depletion of Arf6 leads to intracellular accumulation of β1 integrin and reduced adhesion and spreading. These findings suggest that Arf6 regulates both endocytosis and recycling of β1 integrins and that BRAG2 functions selectively to activate Arf6 during integrin internalization.

Results

Multiple BRAG2 Splice Isoforms

There are currently 15 recognized Arf GEFs in the human genome, and these can be divided into five subfamilies [2]. The previously described Arf-GEP100 [3] belongs to a new subfamily referred to as the Brefeldin-Resistant Arf GEFs (BRAGs). In this nomenclature, Arf-GEP100 is called BRAG2. We found that immunoprecipitation of Hela cell lysates with a polyclonal BRAG2 antibody yielded two bands, one of 150 kDa and one of 120 kDa (Figure 1A). Database analysis revealed that BRAG2 may exist in as many as four splice isoforms, one of which encodes a larger product with an N-terminal extension of 122 amino acids (Figure 1B; also Figure S1 in the Supplemental Data available with this article online). We were able to amplify a PCR product from HEK293 cell mRNA corresponding to this longer isoform, which we call BRAG2b. When expressed in Hela cells, this longer isoform comigrated with the 150 kDa endogenous protein, whereas Arf-GEP100 (which we now call BRAG2a) comigrated with the 120 kDa band (data not shown). Both proteins were depleted by siRNA-mediated knockdown with an siRNA duplex corresponding to a region shared by both isoforms, thus confirming that these proteins represent endogenous BRAG2a and BRAG2b (Figure 1A). Both isoforms were also detected in a wide variety of other cell lines, suggesting that they are ubiquitously expressed.

Like other Arf GEFs, BRAG2 is expressed at low levels in most cells. We were unable to detect endogenous BRAG2 by immunofluorescene microscopy in any examined cell type, including HEK293, MDCK, A431, BHK, PC12, and Hela cells (data not shown). Therefore, to determine if BRAG2a and BRAG2b are differentially localized, we examined the distribution of epitope-tagged constructs by immunofluorescence microscopy. Surprisingly, we found that both BRAG2a and BRAG2b exhibited a pronounced accumulation in the nucleus in most cell types (Figure S2), although a cytosolic pool was readily apparent, especially for BRAG2b. Both isoforms were exclusively nuclear after treatment of cells with leptomycin B (Figure S2), which blocks the Crm1/exportin1 nuclear-export machinery, suggesting that these proteins cycle between the nucleus and the cytoplasm. Results of a recent nuclear proteomics analysis also confirm the presence of endogenous BRAG2 in Hela cell nuclei [4]. However, immunoprecipitation of endogenous BRAG2 from cytosolic and nuclear fractions of Hela cells revealed that approximately 90% of each isoform is cytosolic at steady state (Figure S2I). It is possible that the nuclear pool appears more prominent by immunofluorescence microscopy because it is concentrated into a smaller volume than the diffuse cytosolic pool, even at low levels of expression.

BRAG2 Isoforms Have In Vivo ARF6 GEF Activity

Previous data indicated that, although BRAG2a exhibited some activity toward Arf1 and Arf5 in vitro, it appeared to prefer Arf6 as a substrate [3]. To evaluate BRAG2 activity in vivo, we used a pull-down assay in which Arf-GTP is recovered from cell lysates based on its affinity for the Arf binding domain of the effector protein GGA3 [5]. HeLa cells were transfected with constructs encoding either BRAG2a or BRAG2b, and Arf6-GTP was precipitated by the use of GST-GGA3 as previously described. In agreement with the previously reported in vitro data, expression of exogenous BRAG2a or BRAG2b resulted in increased levels of Arf6-GTP (Figure 1C). As expected, the GEF activity of BRAG2 was dependent upon the Sec7 catalytic domain and could be disrupted by a point mutation substituting the conserved catalytic glutamate residue with lysine (BRAG2aE498K). Importantly, the Arf-GEF activity of BRAG2a

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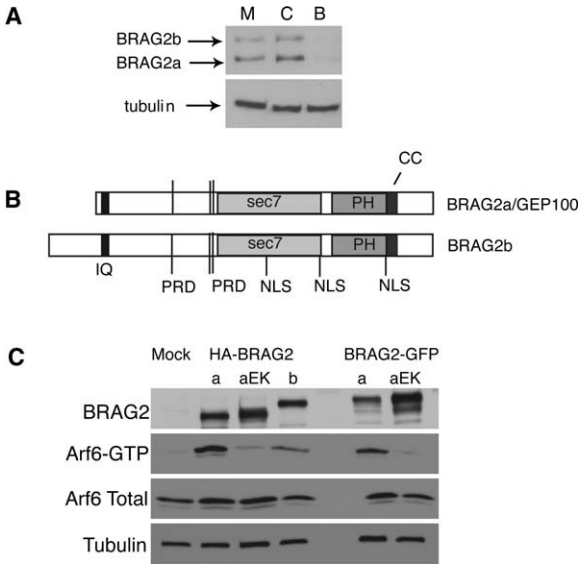

Figure 1. BRAG2 Exists in Two Isoforms that Each Activate ARF6 In Vivo

(A) Hela cells were either mock-transfected (M) or tranfected for 48 hr with scrambled siRNA duplex (C) or a specific duplex targeting a sequence shared by both isoforms (B). Endogenous BRAG2 was then concentrated by immunoprecipitation and detected by immunoblotting with the same antibody. Lysates were probed with α-tubulin antibody to demonstrate equal protein levels in each sample.

(B) Domain organization of BRAG2a and BRAG2b. The two isoforms share a putative calmodulin binding IQ domain (IQ), three proline-rich domains (PRD), three predicted nuclear-localization signals (NLS), the catalytic Arf-GEF domain (sec7), a pleckstrin homology domain (PH), and a coiled-coil domain (CC).

(C) Both BRAG2 isoforms exhibit ARF6 GEF activity in vivo. HeLa cells were cotransfected with HA-ARF6 and one of the following: empty vector (mock), HA-BRAG2a (a), HA-BRAG2b (b), BRAG2a-GFP, or one of the catalytically inactive mutants HA-BRAG2aE498K and BRAG2aE498K-GFP (aEK). GTP bound Arfs were precipitated from cell lysates by a pull-down assay with GST-GGA3 as previously described [5] and probed for Arf6 with antibody to the HA tag. Expression levels of the various BRAG2 constructs, total HA-ARF6, and endogenous α-tubulin in total cell lysates are shown.

did not appear to be disrupted by the presence of either an N-terminal HA tag or a C-terminal GFP tag (Figure 1C). Furthermore, in keeping with their role as Arf6 GEFs, we found that ectopic expression of either BRAG2a or BRAG2b resulted in actin-cytoskeleton alterations, including disruption of stress fibers and changes in cell shape (Figure S3). Finally, as we have previously shown for another Arf6 GEF, ARNO [6], treatment of cells with phorbol esters led to recruitment of both BRAG2a and BRAG2b to the cell cortex and enhanced their effects on the actin cytoskeleton (Figure S3).

A Role for BRAG2 in Cell Adhesion

Arf6 has been reported to mediate the recycling of integrins from an endosomal compartment to the plasma membrane [1], and expression of dominant-negative Arf6 impairs both cell spreading [7] and cell migration [8, 1, 5, 9]. To determine if BRAG2 regulates Arf6 activation in the context of cell adhesion, we measured attachment and spreading in cells where BRAG2 expression was reduced by using RNAi. As described above, we reduced expression of both BRAG2a and BRAG2b by using a single siRNA duplex corresponding to a sequence shared by both isoforms (see Experimental Procedures). Expression of both BRAG2a and BRAG2b was typically reduced by 85% under these conditions (Figure 2A).

To determine if knockdown of BRAG2 altered cell adhesion, we measured the binding of cells to immobilized fibronectin in a dose-response assay. Equivalent numbers of cells were plated onto microtiter wells coated with fibronectin at concentrations ranging from 0–10 ug/ml. To our surprise, we found that cells in which BRAG2 was depleted actually bound fibronectin more efficiently than control cells, especially at moderate fibronectin concentrations (Figure 2B). Interestingly, this effect was specific for BRAG2; knockdown of ARNO, which is also expressed in Hela cells and can also activate Arf6, had no detectable effect on cell adhesion.

Similarly, we found that cells in which BRAG2 was knocked down spread more rapidly than controls on fibronectin-coated substrates (Figures 3A and 3C). Quantitative analysis of cells attached for 30 min indicated that the mean area of the BRAG2 knockdowns was more than twice that of control cells and that the range of cell diameters was clearly broader (Figures 3B and 3D).

These observations suggested that reduced BRAG2 expression leads to either increased levels of integrins on the cell surface or enhanced activation of existing surface integrins. Because the primary fibronectin binding integrin in Hela cells is $\alpha5\beta1$, we quantitated the surface expression of the endogenous $\beta1$ integrin subunit (CD29) by flow cytometry by using a pan-$\beta1$ monoclonal antibody (MEM101A) that is not sensitive to conformation or ligand binding. Over four separate experiments, knockdown of BRAG2 resulted in an average 51% ($\pm$ 6.8%) increase in surface levels of $\beta1$ relative to controls (Figure 4B). Immunoblotting of cell lysates demonstrated that overall expression of the $\beta1$ subunit was unaffected by BRAG2 knockdown (data not shown), indicating that the observed changes resulted from redistribution of the integrin from an intracellular pool to the cell surface. Taken together, these data indicate that BRAG2 regulates the trafficking of $\beta1$ integrins and that this can have a quantitatively significant impact on cell attachment and spreading.

Knockdown of Arf6 Reduces Surface Integrin Levels and Inhibits Cell Spreading

Because BRAG2 is an activator of Arf6, BRAG2 knockdown should result in reduced levels of Arf6-GTP at the plasma membrane. In principle, expression of dominant-negative Arf6 should have a similar effect, by binding and sequestering endogenous GEFs. However, as described above, the dominant-negative Arf6 mutant Arf6T27N has previously been shown to inhibit integrin recycling [1], cell spreading [7], and migration [5], exactly the opposite of what we observed when BRAG2 levels were reduced. However, the interpretation of experiments utilizing dominant-negative Arf6 constructs may be complicated by Arf6T27N sequestering GEFs that normally activate other Arfs, or by binding to other non-GEF proteins that may alter cell attachment. To circumvent this problem, we used RNAi to knock down

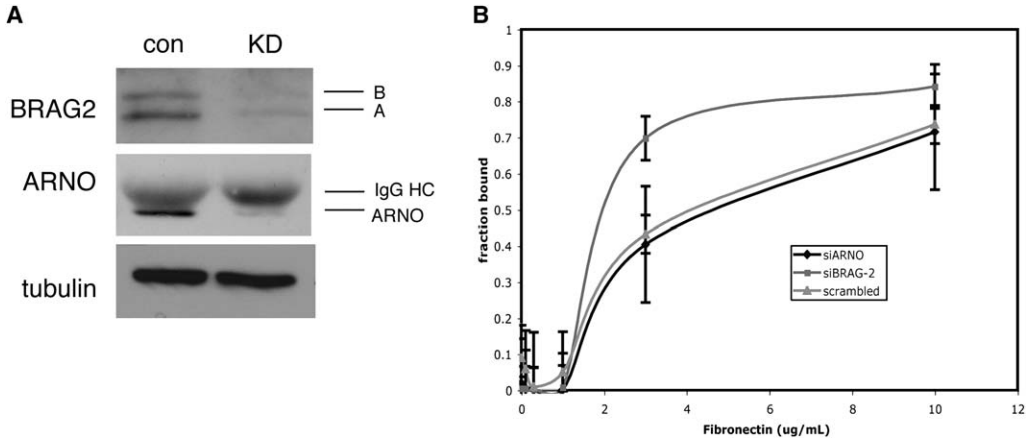

Figure 2. Knockdown of BRAG2 Enhances Binding of Cells to Fibronectin

(A) Knockdown of BRAG2 or ARNO by RNAi. HeLa cells were treated for 48 hr with RNA duplexes, and knockdown was confirmed by immunoprecipitation followed by immunoblotting. Endogenous ARNO runs immediately below the IgG heavy chain on SDS PAGE gels.

(B) Cell binding to fibronectin. HeLa cells transfected with scrambled (triangle), ARNO (diamond), or BRAG2 (square) RNA duplexes were plated onto 96-well plates coated with fibronectin concentrations ranging from 0 to 10 μg/ml, and adherent cells were then quantitated as described in the Experimental Procedures. Error bars indicate the mean ± SE for triplicate determinations.

expression of endogenous Arf6. This knockdown was selective because Arf6 expression was typically reduced by more than 80%, whereas expression of endogenous Arf1 was not detectably altered (Figure 4A). Intriguingly, analysis of these cells by flow cytometry revealed that surface expression of β1 integrin was reduced by 34% (± 5.9%, n = 4), in contrast to the elevated surface levels in cells where BRAG2 was knocked down

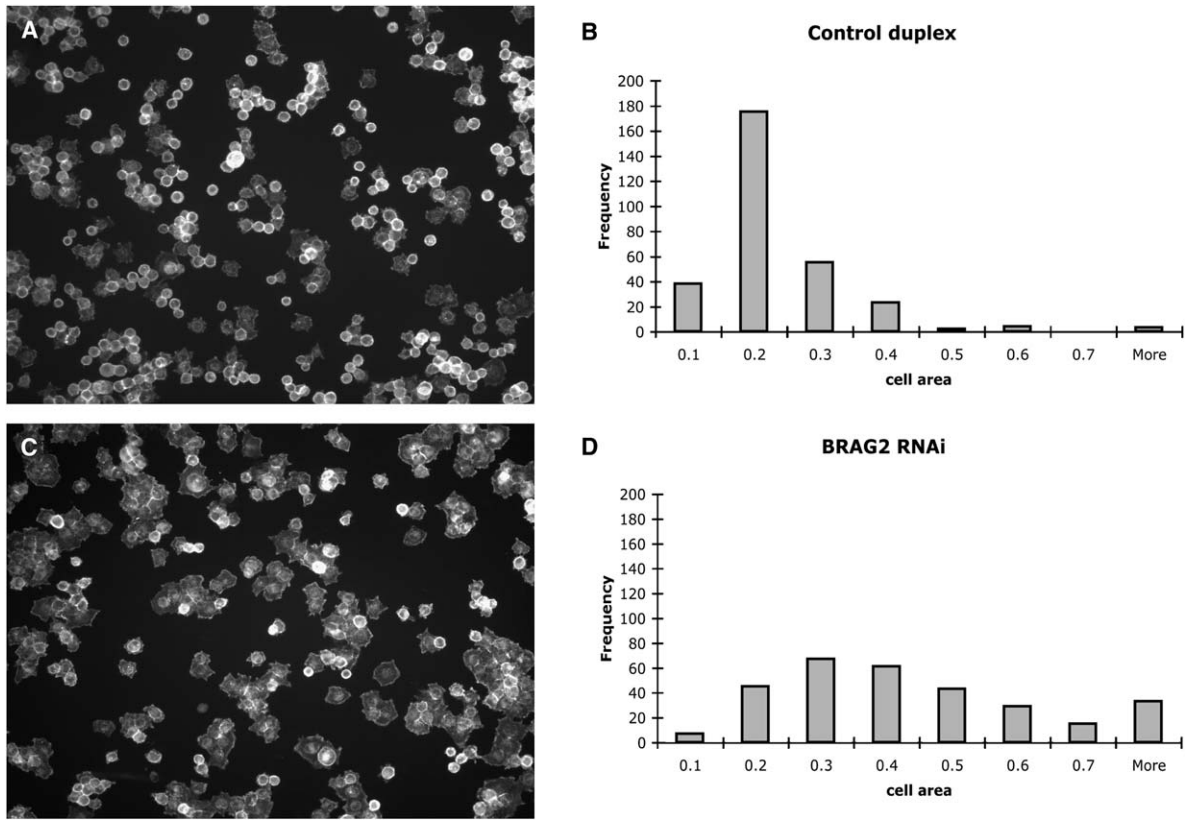

Figure 3. Depletion of BRAG2 Enhances Cell Spreading on Fibronectin-Coated Substrates

HeLa cells treated with either control siRNA duplexes (A and B) or BRAG2-specific duplexes (C and D) were harvested nonenzymatically and replated onto fibronectin-coated coverslips for 15 min as described in the Experimental Procedures. Cells were then fixed and stained for filamentous actin (A and C). For each condition, the area of 300 individual cells was quantitated with ImageJ software, and the distributions are shown in (B) and (D).

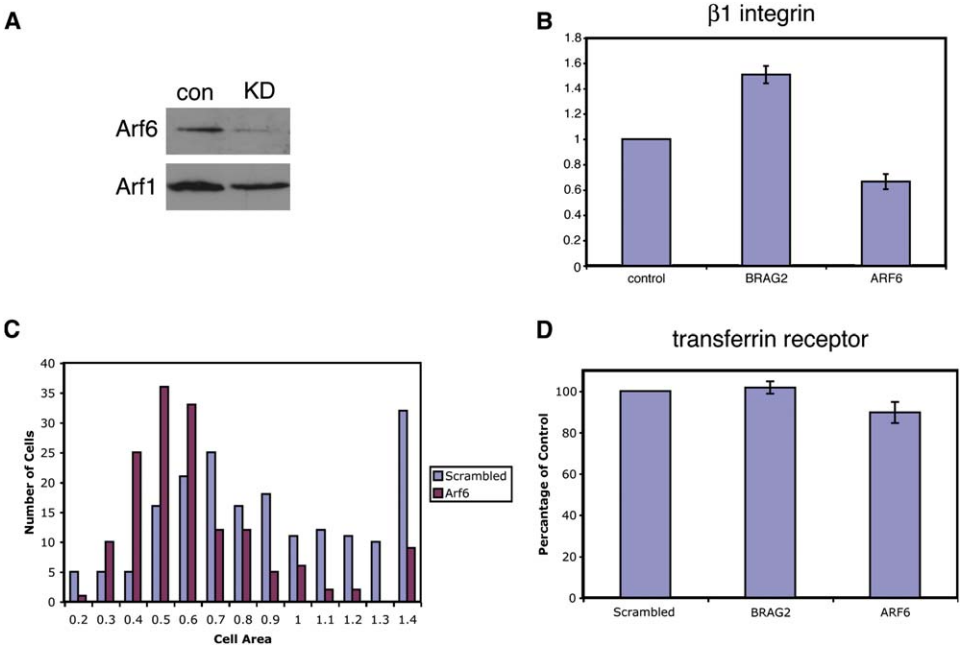

Figure 4. Depletion of BRAG2 and Arf6 Have Opposing Effects on Cell-Surface Expression of β1 Integrin but Not Transferrin Receptor

(A) Selective depletion of Arf6 by RNAi. Hela cells were treated with either scrambled oligonucleotide (con) or Arf6-specific siRNA duplex (KD). Lysates were then probed with antibodies specific for endogenous Arf6 or Arf1.

(B) FACS analysis of surface β1 integrin expression. HeLa cells were transfected with RNA duplexes as indicated and harvested 48 hr later as described in Figure 2. Cells were then incubated with PE-conjugated anti-β1 integrin antibody MEM101A and analyzed by flow cytometry. Values are normalized to the mean fluorescence intensity of control cells. Error bars indicate the mean ± SE of four independent determinations.

(C) Cells transfected with control RNA duplex (blue) or Arf6-specific duplex (red) were harvested and replated on fibronectin-coated coverslips as described in Figure 3. Cell areas were quantitated 60 min after replating.

(D) Cells were treated as in (B) but were labeled with PE-conjugated anti-transferrin receptor antibody (CD71). Error bars indicate the mean ± SE of four independent determinations.

(Figure 4B). As described above for the BRAG2 knockdown, total β1 expression was unchanged under these conditions, suggesting that β1 integrins were sequestered inside the cell. In agreement with this observation, we found that cells expressing reduced levels of Arf6 spread less efficiently on fibronectin-coated substrates (Figure 4C). Finally, no change in the level of surface transferrin receptor was observed under these conditions (Figure 4D), indicating that the effects of either BRAG2 or Arf6 depletion are cargo specific. Taken together with the results described above, these findings suggest that Arf6 functions at multiple sites in the endocytic/recycling system and that BRAG2 selectively regulates Arf6 activation in the endocytic process.

Discussion

Previous work has shown that β1 integrins internalized from the cell surface enter an endosomal compartment that is enriched for Arf6 [1, 10]. In addition, expression of dominant inhibitory Arf6 mutants impairs recycling of this endosomal integrin pool to the plasma membrane, suggesting that Arf6 activation is necessary for integrin recycling [1]. Here we show that siRNA-mediated knockdown of Arf6 has a similar effect in that it reduces surface expression of β1 integrins and inhibits both cell attachment and spreading on fibronectin-coated substrates. Because overall levels of β1 were unchanged under these conditions, we interpret these results to indicate that depletion of Arf6 impairs β1 recycling.

In contrast, although our experiments clearly demonstrate that BRAG2 potently activates Arf6 in vivo, we found that depletion of endogenous BRAG2 leads to accumulation of β1 integrin at the cell surface, and this accumulation results in more efficient binding of cells to fibronectin and an increased rate of spreading. This observation suggests that internalization of β1 integrins is mediated, at least in part, by BRAG2, through its activation of Arf6 at the plasma membrane.

Why does depletion of BRAG2 have the opposite effect from depletion of Arf6 itself? In the secretory pathway, Arf1 is thought to function at multiple locations to generate different classes of carrier vesicles, and accumulating evidence indicates that different Arf1 GEFs control its activation at each site [11–16]. It is likely that a similar scenario exists for Arf6 in the endocytic pathway, with one subset of GEFs functioning at the plasma membrane and another on the surface of endosomes. Thus, knockdown of a specific Arf6 GEF would impair only the transport step(s) mediated by that GEF, rather than all steps along the pathway that are Arf6-dependent. The intracellular accumulation of β1 integrin after Arf6 depletion may reflect a more rigid requirement for Arf6 in recycling than in endocytosis. Although other GEFs presumably activate Arf6 on the surface of endosomes to promote recycling, their identity remains unknown. Because the PH domains of EFA6 and ARNO/cytohesin subfamily members preferentially bind $PI(4,5)P_2$ and $PI(3,4,5)P_3$, respectively, it seems likely that these GEFs function primarily at the plasma membrane, which

is enriched in these lipid species. Our observation in this study that knockdown of ARNO did not affect cell adhesion supports this hypothesis. However, other BRAG subfamily members, such as BRAG1 or BRAG3, which are relatively uncharacterized, remain possibilities.

How does Arf6 regulate integrin internalization? Surprisingly little is known about the mechanisms by which integrins are internalized. Both αvβ5 [17] and α5β1 integrins [18, 19] have been reported to localize at least partially in clathrin-coated pits, and αvβ5 endocytoses bound vitronectin in a clathrin-dependent manner [17]. Arf6-mediated activation of phosphatidylinositol 4-P 5-kinase (PIP5K) isoforms has been shown to promote the assembly of clathrin and the AP-2 adaptor complex onto the plasma membrane [20, 21]. Moreover, bacterial pathogens of the genus *Yersinia* are internalized via their interaction with β1 integrins, and recent evidence indicates that bacterial uptake requires the recruitment and activation of PIP5K-α by Arf6 [22]. Whether Arf6 also regulates integrin internalization via its activation of PIP5K remains to be determined.

It will also be interesting to determine if BRAG2 and Arf6 regulate the internalization of other molecules at the cell surface. For example, the *Drosophila* BRAG2 ortholog, Schizo, has been reported to regulate neuronal pathfinding in the developing central nervous system by controlling the surface levels of the repulsive ligand Slit on midline glial cells [23]. Because the Slit/Robo signaling pathway is conserved in mammals [24], BRAG2 may play a similar role in mammalian development.

Conclusions

The number of GEFs (15) and GAPs (17) that control Arf activation far exceeds the number of Arfs themselves, and a major challenge in this field has been to assign specific cellular functions to each of these regulatory molecules. Among the Arf GEFs, the BRAGs have been relatively uncharacterized. Here we show that BRAG2 exists in two ubiquitously expressed isoforms that both activate Arf6 in vivo. These proteins appear to function primarily in the cell periphery, where they regulate the surface level of β1 integrins and, consequently, cell adhesion. The specific mechanisms by which Arf6 may regulate integrin endocytosis remain to be explored.

Experimental Procedures

DNA Constructs

BRAG2a (KIAA0763) cDNA was obtained from the Kasuza DNA Research Institute and subcloned into EcoRI/NotI sites of pRC-HA. This vector was used as a template for site directed PCR mutagenesis to produce catalytically inactive BRAG2a (E498K). cDNA encoding the extended N terminus of BRAG2b was amplified by RT-PCR from HEK293 mRNA with the following primers: 5′-GCCGCCAT GTGGTGCCTGC-3′ and 5′-CTGGCGAAACGCCGTCTGGATGG-3′. This cDNA was subcloned into the pRC-HA-BRAG2a vector to produce pRC-HA-BRAG2b. BRAG2a-GFP was subcloned into the HindIII/BamHI sites of pRK7.

Cell Culture and Transfections

HeLa cells were maintained in complete DME (10% FBS) with antibiotics at 37ºC and with 5% CO_2. Cells were transfected with DNA plasmids via Fugene6 as recommended by the manufacturer (Roche). For RNAi, approximately 117 nM final concentration of siRNA duplex was delivered to cells by the use of 10 μl Oligofectamine (Invitrogen)/35 mm dish in a total volume of about 1.7 ml. The BRAG2 siRNA duplex corresponded to the sequence 5′-AGAAC UCGGUGACGUACAG-3′ and was obtained from Dharmacon. The control duplex (5′-UCGACUGUGGAUUGGCAUU-3′) was obtained from Ian Macara (The University of Virginia).

Immunofluorescence and Image Analysis

Cells were grown on glass coverslips and fixed with 4% paraformaldehyde 48 hr after transfection. Images were acquired with a Nikon Eclipse E800 microscope equipped with a RETIGA digital camera (Q-Imaging) and with Adobe Photoshop software.

Immunoprecipitation and Immunoblotting

Polyclonal rabbit anti-BRAG2 antibody, raised against residues 153–176 of BRAG2a as described previously [3], was obtained from Vladimir Marshansky (Massachusetts General Hospital) and used for immunoprecipitation at 1/500 dilution. At least 1.8 mg of total HeLa cell protein was required to detect BRAG2 isoforms by immunoprecipitation. Immunoprecipitation and immunoblotting were performed as described by Hansen and Casanova [25]. For immunoblotting, a 1/1000 dilution of rabbit anti-BRAG2, a 1/500 dilution of mouse anti-Arf6 (8A6-2 from Sylvain Bourgoin, CHUL research center, Quebec), and a 1/2000 dilution of polyclonal anti-Arf1 and 1/10,000 of mouse anti-α-tubulin (DM1A Sigma) were used.

Cell-Adhesion Assays

Triplicate wells of microtiter plates were coated with fibronectin in concentrations ranging from 0.1 to 10 μg/ml, and nonspecific binding sites blocked with 10 mg/ml heat-denatured BSA for 1 hr. HeLa cells were harvested nonenzymatically by incubation in PBS containing 4 mM EDTA and 4 mM EGTA, washed in serum-free DMEM, and aliquoted into wells for 15 min at 5×10^5 cells/well. After removal of nonadherent cells by washing, remaining cells were fixed in 4% paraformaldehyde and incubated with 1% crystal violet for 15 min. After extensive washing, the dye was extracted with 1% deoxycholate, and the OD_{595} was quantitated in a microplate reader.

For spreading assays, cells were harvested as above and replated onto glass coverslips coated with 10 μg/ml fibronectin for 15 min or 30 min at 37ºC. Cells were then fixed and permeablized, and random fields of cells were imaged with a 20× phase-contrast objective lens. The diameters of 300 cells per condition were measured with Image J software (NIH), and the diameters were sorted into bins with Excel.

Flow Cytometry

Cells were harvested as described above and washed in PBS containing 0.2% BSA, and 1×10^5 cells per sample were stained without fixation at 4ºC. For detection of surface β1 integrin, PE-conjugated anti-CD29 IgG (Caltag) was used at 1:400. Nonspecific labeling was determined with a PE-labeled isotype control antibody. After samples were fixed in 4% paraformaldehyde, the mean fluorescence intensity of each sample was quantitated with a Becton Dickinson FACScalibur desktop analyzer.

Supplemental Data

Supplemental Data include three figures and are available with this article online at http://www.current-biology.com/cgi/content/full/16/3/315/DC1/.

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