An N-terminally acetylated Arf-like GTPase is localised to lysosomes and affects their motility

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Accepted 24 February 2006
Journal of Cell Science 119, 1494-1503 Published by The Company of Biologists 2006
doi:10.1242/jcs.02958

Summary

Small GTPases of the Arf and Rab families play key roles in the function of subcellular organelles. Each GTPase is usually found on only one compartment and, hence, they confer organelle specificity to many intracellular processes. However, there has so far been little evidence for specific GTPases present on lysosomes. Here, we report that two closely related human Arf-like GTPases, Arl8a and Arl8b (also known as Arl10b/c and Gie1/2), localise to lysosomes in mammalian cells, with the single homologue in *Drosophila* cells having a similar location. Conventionally, membrane binding of Arf and Arl proteins is mediated by both an N-terminal myristoyl group and an N-terminal amphipathic helix that is inserted into the lipid bilayer upon activation of the GTPase. Arl8a and Arl8b do not have N-terminal myristoylation sites, and we find that Arl8b is instead N-terminally acetylated, and an acetylated methionine is necessary for its lysosomal localization. Overexpression of Arl8a or Arl8b results in a microtubule-dependent redistribution of lysosomes towards the cell periphery. Live cell imaging shows that lysosomes move more frequently both toward and away from the cell periphery, suggesting a role for Arl8a and Arl8b as positive regulators of lysosomal transport.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/??/????/DC1

Key words: Arf family, GTPase, Lysosome, N-terminal acetylation

Introduction

The organisation of internal membranes requires that many peripheral membrane proteins are accurately recruited to specific organelles from the cytosol. These peripheral proteins mediate vesicle transport between organelles, or interactions between organelles and the cytoskeleton. In general, their recruitment reflects the recognition of activated GTPases or specialised lipid species, such as phosphoinositides, which are present on only one organelle (Behnia and Munro, 2005; Munro, 2004). These GTPases are members of two large families, the Rab and Arf families. Like many other members of the Ras superfamily of small GTPases, the Rabs are attached to membranes via C-terminal prenyl groups. In contrast, the Arf family members are distinguished by having an N-terminal amphipathic helix and, in most cases, an N-terminal myristoyl group (Pasqualato et al., 2002; Pfeffer, 2001; Zerial and McBride, 2001). The hydrophobic side of the amphipathic helix is buried when Arf is in the GDP-bound form, but upon nucleotide exchange there is a conformational change unique to Arfs that displaces the helix (Goldberg, 1998; Pasqualato et al., 2002). The hydrophobic side of the displaced helix interacts with the lipid bilayer, resulting in the GTP-bound form being tightly associated with the bilayer. In this membrane-associated form, Arf can then recruit effectors until GTP hydrolysis is induced by a GTPase-activating protein (GAP) and, hence, Arf returns to the GDP-bound form and falls off the membrane.

The best characterised members of the Arf family are Sar1, Arf1 and Arf6, which are localised to the ER, Golgi and plasma membrane, respectively. Sar1 recruits the COPII vesicle coat, and Arf1 recruits the COPI, AP1 and GGA coat proteins as well as further effectors proposed to contribute to membrane traffic (Donaldson et al., 2005; Lee et al., 2004; Nie et al., 2003). Arf6 appears to regulate cytoskeletal dynamics and membrane traffic at least in part, by recruiting a PI 5-kinase that synthesises PtdIns(4,5)P_2 (Aikawa and Martin, 2003; Krauss et al., 2003). However, in addition to these Arf family members, genome sequencing has revealed a number of 'Arf-like' GTPases that share with Sar1 and the Arfs an N-terminal amphipathic helix, and the 'interswitch' region responsible for the conformational change that displaces the helix (Kahn et al., 2006; Li et al., 2004; Pasqualato et al., 2002). There are 22 such Arls in humans, some of which are well-conserved in evolution, with eight forms having clear homologues in *Drosophila*. The function of most is presently unknown, but Arl1 and ARFRP1 (Arl3 in yeast) have been shown to be localised to the Golgi, where they form a pathway that results in the recruitment of coiled-coil proteins to the Golgi via a direct interaction between Arl1-GTP and the GRIP domain at the C-terminus of the coiled-coil protein (Gangi Setty et al., 2003; Munro, 2005; Panic et al., 2003). In addition, Arl2 and Arl3 appear to regulate the activity of a tubulin-folding cofactor (Burd et al., 2004). Many of the Arl proteins have a glycine residue at position 2 that is part of the consensus for recognition by N-myristoyltransferase, which modifies the α-amino group of the conserved glycine following removal of the initiator methionine by methionine aminopeptidase (Bradshaw et al., 1998; Maurer-Stroh et al., 2002). Hence, probably most of the Arls are myristoylated, and this has been confirmed in

most cases examined. However, one of the Arls, ARFRP1/Arl3 that is found on the Golgi, lacks a glycine at position 2 and, instead, has a well-conserved tyrosine or phenylalanine. It has been recently found that this results in the initiator methionine being left uncleaved, and the N-terminus being acetylated by the NatC complex, a minor N-terminal acetylation complex that acts on proteins with phenylalanine, tyrosine, isoleucine, leucine or tryptophan at position 2 (Behnia et al., 2004; Setty et al., 2004). These studies were done with yeast Arl3, but mammalian ARFRP1 is likely to be modified in the same way because the mammalian orthologue of NatC apparently has a similar substrate specificity to the yeast acetyltransferase (Polevoda and Sherman, 2003).

In this paper, we examine two previously uncharacterised human Arls, Arl8a and Arl8b, that also lack a glycine at position 2. Arl8a and Arl8b are very closely related proteins (91% identity) that were originally identified from the human genome sequence (Kahn et al., 2006; Li et al., 2004; Pasqualato et al., 2002). Phylogenetic analysis indicates that Arl8a and Arl8b define an Arl subfamily that has a single member in many species, including *Drosophila melanogaster*, *Caenorhabditis elegans*, filamentous fungi, plants, *Dictyostelium* and *Trypanosoma cruzi*. Interestingly, there is no homologue in the yeast *Saccharomyces cerevisiae*, indicating that Arl8 is an ancient GTPase that budding yeast have lost during evolution. We show that Arl8a and Arl8b are localised to lysosomes and that Arl8b is N-terminally acetylated, with mutations that perturb the N-terminus, causing a loss of lysosomal localisation.

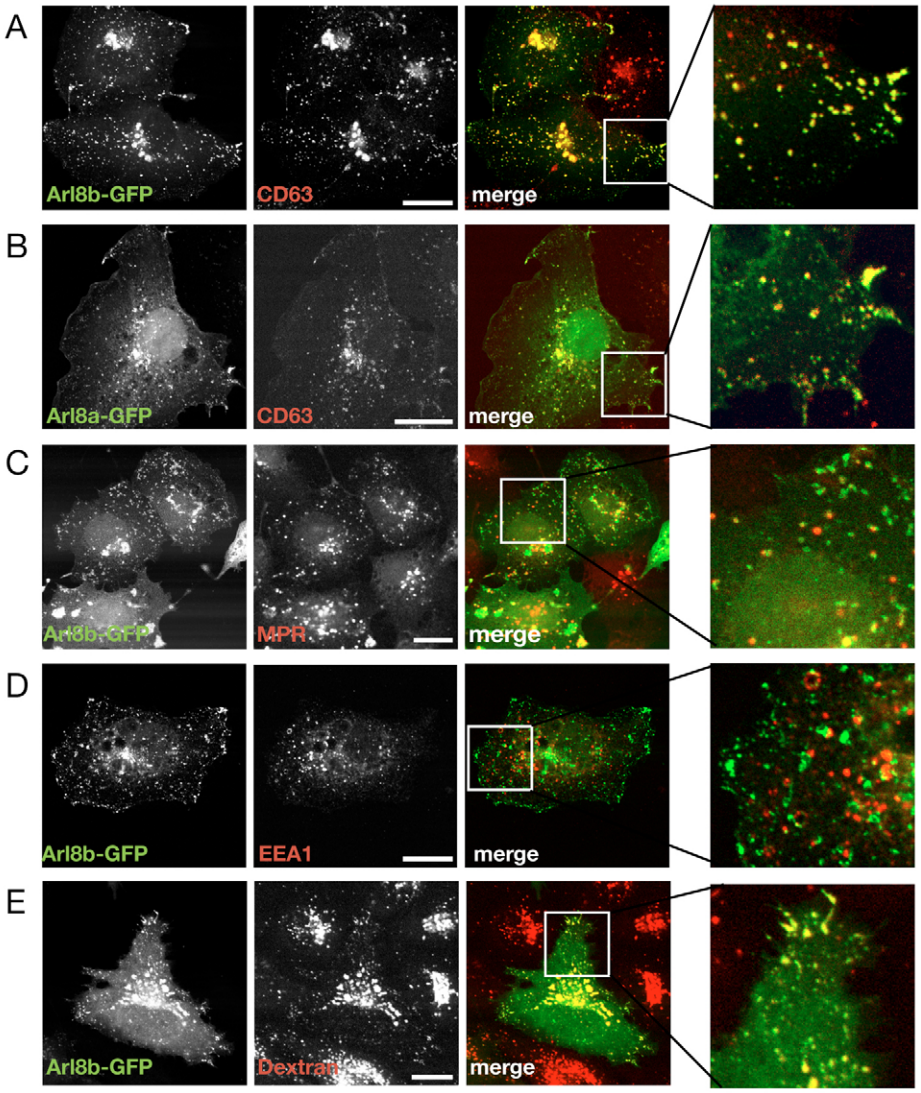


Fig. 1. Arl8a and Arl8b localise to lysosomes. (A-D) Confocal micrographs of COS cells transfected with plasmids expressing either Arl8a-GFP or Arl8b-GFP from a CMV promoter. After fixation and permeabilization, the cells were labelled with antibodies against the indicated endogenous proteins CD63 (lysosomes), MPR (cation-dependent mannose 6-phosphate receptor, late endosomes) and EEA1 (early endosomes). (E) Confocal micrographs of NRK cells expressing Arl8b-GFP. Lysosomes were labelled with endocytosed dextran-tetramethylrhodamine using a four hour pulse at 1 mg/ml, followed by a 20-hour chase. Bars, 10 μm.

Results

Arl8a and Arl8b localise to lysosomes in mammalian cells

To examine human Arl8a and Arl8b in more detail, we tagged both proteins at the C-terminus and expressed them in COS cells. With both GFP and HA epitope tags, Arl8a and Arl8b showed a punctate distribution displaying extensive colocalization with several lysosomal markers, such as CD63 and LAMP2 (Chen et al., 1985; Fraile-Ramos et al., 2001) (Fig. 1A,B and Fig. 2B). Both proteins showed a limited overlap with the late endosomal markers Rab7 and the cation-dependent mannose-6-phosphate receptor (Rink et al., 2005) (Fig. 1C and data not shown), and no overlap with EEA1, a marker for early endosomes (Stenmark et al., 1996) (Fig. 1D). A lysosomal localization of Arl8b was also observed in rat NRK cells. When these cells were treated with fluorescent dextran as a fluid-phase endocytic marker, using a pulse followed by chase to label terminal endocytic compartments, the dextran accumulated in Arl8b-positive structures (Fig. 1E). Thus, Arl8a and Arl8b are predominantly localised to lysosomes, with some colocalization with late endosomal markers that might reflect the cycle of fusion and resolution between lysosomes and late endosomes (Bright et al., 2005). As such, Arl8a and Arl8b are the first small GTP-binding proteins reported to be localised to lysosomes.

Some of the human Arls appear to have arisen only recently

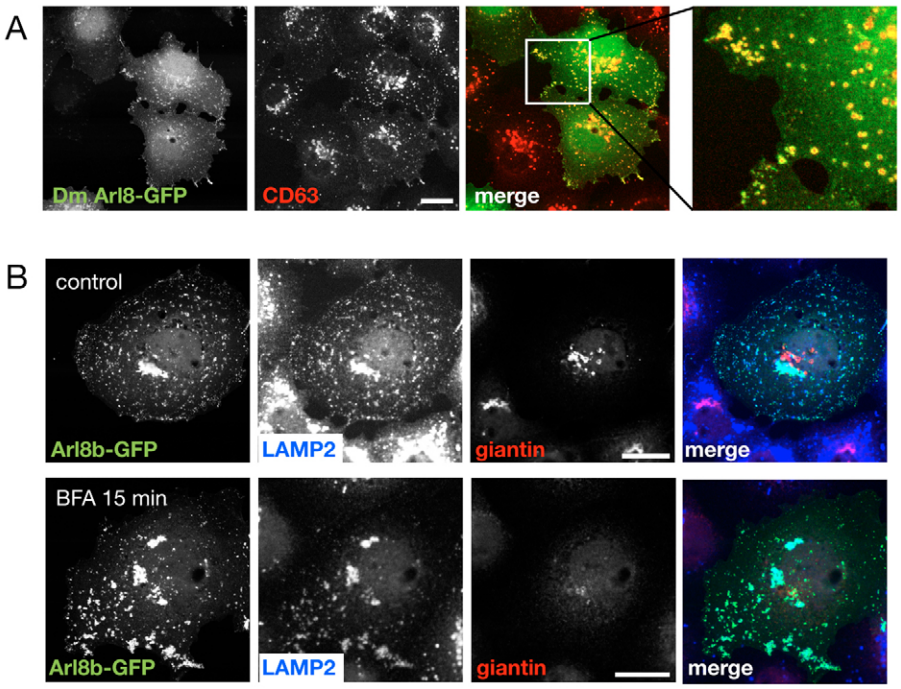


Fig. 2. Arl8 targeting is evolutionarily conserved but brefeldin A resistant. (A) Confocal micrographs of COS cells expressing the *Drosophila* Arl8 homologue (CG7891) with GFP at the C-terminus (Dm Arl8-GFP), and after fixation and permeabilisation labelled with antibody against the lysosomal protein CD63. (B) Confocal micrographs of COS cells expressing Arl8b-GFP and either untreated, or incubated in 5 μg/ml brefeldin A for 15 minutes, before fixation and labelling with the indicated antibodies to residents of the lysosome (LAMP2) and the Golgi (giantin). Bars, 10 μm.

upon vertebrate evolution, but Arl8 is one of the eight human Arls that are conserved outside vertebrates (Li et al., 2004; Pasqualato et al., 2002). When *Drosophila* Arl8 (CG7891) was expressed in COS cells as a GFP fusion it also localised to lysosomes, suggesting that the targeting of the protein is well conserved (Fig. 2A). The recruitment of Arfs to the Golgi is inhibited by the fungal metabolite brefeldin A, which blocks the activity of a subset of Arf GEFs (Renault et al., 2003). We thus examined the effect of brefeldin A in COS cells but found that it did not change the lysosomal targeting of Arl8a or Arl8b, although the Golgi marker giantin was perturbed as expected (Fig. 2B and data not shown). This indicates that Arl8a and Arl8b and their GEF or GEFs are brefeldin-insensitive.

Arl8b is N-terminally acetylated

Arl8a and Arl8b lack a glycine at position 2 and, therefore, cannot be myristoylated (Fig. 3A,B). Instead, the second residue of Arl8a is an isoleucine and that of Arl8b – and of homologues in metazoans – a leucine. These residues at the second position make Arl8a and Arl8b potential substrates for the NatC N-terminal acetyltransferase (Polevoda and Sherman, 2003). To investigate its N-terminal acetylation, HA-tagged Arl8b was immunoprecipitated from transfected COS cells and following SDS gel electrophoresis, tryptic peptides were analysed by mass-spectrometry. We identified a peptide with a mass corresponding to an acetylated N-terminus (Fig. 3C), showing that Arl8b is N-terminally acetylated.

A number of cytosolic proteins, including some GTPases, associate with membranes via palmitoyl groups attached to internal cysteine residues (Smotrys and Linder, 2004). Arl8a and Arl8b contain three cysteines (residues 158, 159 and 164) that are conserved in homologues from humans to plants but do not occur in other Arls (Pasqualato et al., 2002). We thus asked whether the role of the N-terminal myristoyl group was performed in Arl8b by a palmitoyl group attached elsewhere. However, when Arl8b-HA was expressed in COS cells it was not labelled with [^{3}H]palmitate under conditions where labelling of a known palmitoylated protein (linker for activation of T cells, LAT) (Zhang et al., 1998)] could be readily detected (Fig. 3D).

An acetylated methionine and the hydrophobic face of the amphipathic helix are required for membrane targeting, but not lysosomal specificity

To investigate the role of the N-terminus in targeting of Arl8b, we examined the effect on localisation of several mutations in this region (Fig. 4A). Replacement of the leucine in position 2 with alanine resulted in a loss of lysosomal localisation, and instead a diffuse cytoplasmic distribution (Fig. 4B). Sequencing of the N-terminal tryptic peptide from this mutant by tandem mass spectrometry indicated that the methionine had been removed by methionine aminopeptidase, as expected for a protein with a small residue in the second position (Bradshaw et al., 1998). Nonetheless, the protein was still acetylated, presumably by the mammalian NatA complex, which acts on many proteins following the removal of the N-terminal methionine (Polevoda and Sherman, 2003). This implies that the N-terminus of the protein is crucial for its localisation. When the leucine in position 2 was replaced with the large hydrophobic residue phenylalanine the methionine was, as expected, left intact and the protein acetylated, presumably by the mammalian NatC orthologue (Polevoda and Sherman, 2003). This form was targeted to lysosomes, suggesting that it is the N-terminally acetylated methionine that is crucial for targeting rather than the precise structure of the residue in the second position (Fig. 4C).

Since Arl8 appears different from most other Arls in lacking an N-terminal myristoyl group, we also investigated the role of the N-terminal amphipathic helix in membrane binding. Mutation to alanine of three hydrophobic residues predicted to lie on one face of an α-helix formed from the N-terminal region

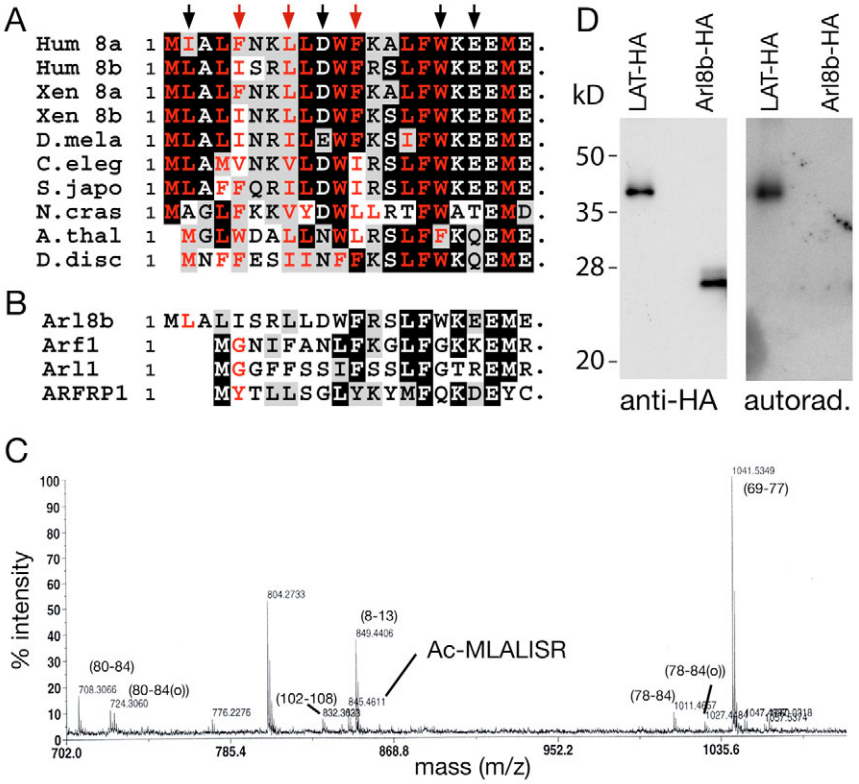


Fig. 3. The N-terminus of Arl8b is acetylated. (A) Alignment of human Arl8a and Arl8b with their relatives from *Xenopus tropicalis*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Schistosoma japonicum*, *Arabidopsis thaliana*, *Neurospora crassa* and *Dictyostelium discoideum*. Sequences were aligned with CLUSTAL W, and shaded in cases where more than half of the residues are related (grey) or identical (black). Hydrophobic amino acids are shown in red, arrows indicate the mutants analysed with combined point mutations in hydrophobic residues (red), or in individual conserved residues (black). (B) Alignment of the N-termini of human Arl8b, Arl1, Arf1 and ARFRP1. The second residue is shown in red. (C) MALDI mass spectrum of tryptic peptides from Arl8b-HA immunoprecipitated from transfected COS cells. Peptides with masses corresponding to expected digestion products of Arl8b are indicated with the sequence for the N-terminal peptide, or with residue numbers (o, oxidised). (D) Anti-HA immunoprecipitates from COS cells expressing LAT-HA or Arl8b-HA and labelled with [^{3}H]palmitate. After gel electrophoresis, the precipitates were either probed with anti-HA antibodies or ^{3}H-detected with fluorography.

(I5A, L8A, F12A) did not appear to affect N-terminal acetylation (Fig. 4A). However, it did result in loss of Arl8b targeting to lysosomes (Fig. 4D). Therefore, in addition to the N-terminally acetylated Met 1, the hydrophobic side of the amphipathic helix appears to be required for membrane binding, as is the case for other Arfs and Arls (Antonny et al., 1997).

To determine whether the acetylated N-terminal helix of Arl8b is involved in the specificity of membrane targeting, we expressed in COS cells chimeras of Arl8b and the Golgi-localised GTPase Arf1. Fig. 4E shows that, when the N-terminal helix of Arl8b was replaced with the equivalent part of Arf1, the chimera was still localised to lysosomes. In contrast, the N-terminal helix of Arl8b only gave a Golgi distribution when attached to Arf1 (Fig. 4F). Thus, it appears that the acetylated N-terminal helix is required for membrane association, but organelle specificity is determined by interactions made by the rest of the protein, perhaps by virtue of recognition by a lysosome-associated GEF.

Expression of Arl8a and Arl8b alters the distribution of lysosomes

The lysosomal location of Arl8a and Arl8b raises the question of their function on this compartment. During the course of the above experiments, we noticed that overexpression of Arl8a and Arl8b resulted in a redistribution of lysosomes in both COS and NRK cells. In such cultured cells, lysosomes typically cluster in the perinuclear region with a few additional lysosomes distributed throughout the cytoplasm (Matteoni and Kreis, 1987). However, cells overexpressing Arl8a or Arl8b still had some perinuclear lysosomes but had more lysosomes localised throughout the cell, including many along the cell periphery, a distribution not normally seen in untransfected cells (Fig. 5A). This effect was even more marked in cells as they spread after replating (Fig. 5B).

Although lysosomal transport is not understood in detail, it is known that lysosomes can move bi-directionally along microtubules, using dynein and kinesin motors (Harada et al., 1998; Jordens et al., 2001; Matteoni and Kreis, 1987). When cells expressing Arl8b were treated with nocodazole to depolymerise microtubules, lysosomes at the edge of cells were no longer observed; instead the lysosomes were scattered throughout the cytoplasm in a manner indistinguishable from nocodazole-treated control cells (Fig. 5C). This indicates that microtubule-based motility is required for maintaining the peripheral and perinuclear pools of lysosomes seen in transfected cells.

Study of other Arf family GTPases has identified mutant forms that are locked in GTP-bound or GDP-bound states (Dascher and Balch, 1994). The analogous mutations in Arl8b were examined. Expression of Arl8b with a Q75L mutation, known to prevent GTP hydrolysis in other Arf family members (Lu et al., 2001; Shin et al., 2005), resulted in a distribution and lysosomal phenotype similar to that seen with the wild-type protein (Fig. 5A). However, when wild-type and Arl8b(Q75L) were expressed in *E. coli* they both accumulated with GTP bound to them, suggesting that the intrinsic rate of GTP hydrolysis is low and, hence, overexpression of the wild-type Arl8b is similar to an accumulation of the GTP-locked form. This would also be consistent with the observation that most of the transfected Arl8b accumulates on lysosomes because, by analogy with other Arf GTPases, high-affinity membrane binding would occur only in the GTP-bound state (Goldberg, 1998; Pasqualato et al., 2002). Arl8b(T34N),

A

Mutant	N-terminal peptide mass	MS/MS sequence
Arl8b(L2A)	672.405	Ac-AALISR
Arl8b(L2F)	895.471	Ac-MFALISR
Arl8b(I2A, L8A, F12A)	803.445	Ac-MLALASR

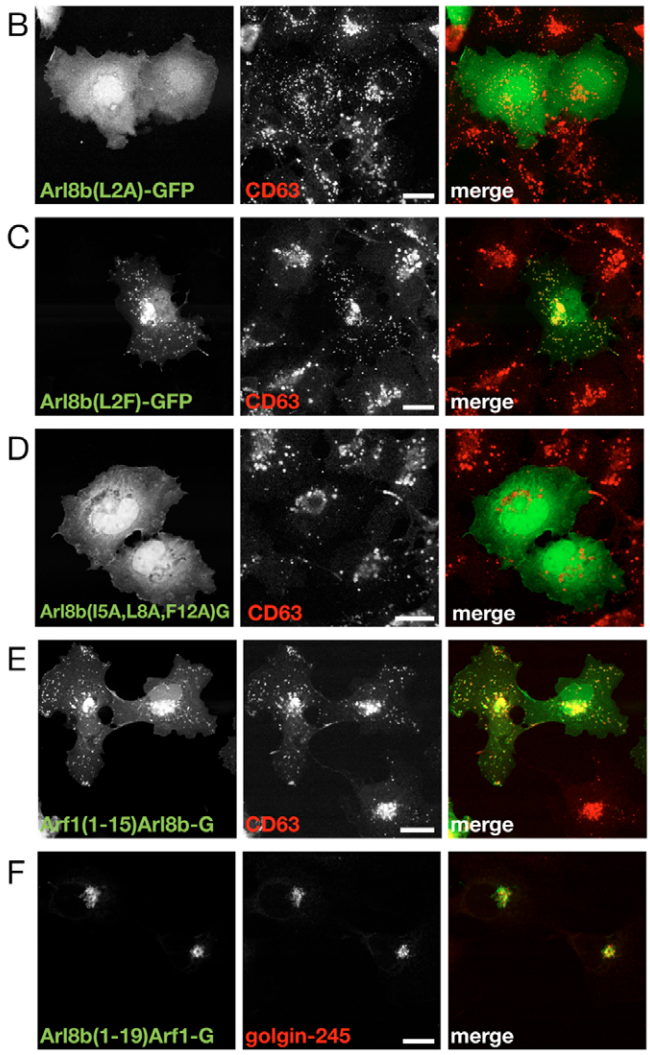


Fig. 4. The N-terminus of Arl8b is required for its lysosomal localisation. (A) Mutant forms of Arl8b, with the corresponding N-terminal peptides obtained after trypsin digestions of HA-tagged forms as in Fig. 3C. The sequence of the N-terminal peptides identified by MALDI were determined by tandem mass spectrometry (MS/MS). The mass of the peptide stated is for the protonated form, with that from Arl8b(L2F) being the form with an oxidised methionine that was more abundant than the native version and hence used for MS/MS sequencing. (B-D) Confocal micrographs of COS cells transfected with plasmids expressing the indicated forms of Arl8b fused to GFP and then, after fixation and permeabilisation, labelled with an anti-CD63 antibody to label lysosomes. (E-F) Confocal micrographs of COS cells transfected with plasmids expressing GFP (-G) fused to the indicated chimeras of Arf1 and Arl8b, and labelled with the indicated antibodies. Replacement of the first 18 residues of Arl8b with the first 15 of Arf1 does not prevent localisation to lysosomes (labelled with CD63), whereas the first 19 residues of Arl8b do not relocalise Arf1 (17-181) from the Golgi (labelled with golgin-245). Bars, 10 μm.

containing a T34N mutation, was predicted to be GDP-bound, and this was found to be the case for *E. coli*-derived material. However, although it did not bind to lysosomes in COS cells, it appeared to have folding problems because it adhered instead to lipid droplets (data not shown). Similar folding problems were found with the other potential dominant-negative mutants we tested (G32A, N130I and D133N), preventing useful interpretation.

Arl8b stimulates lysosomal motility

To investigate further the effects of Arl8a and Arl8b on the distribution of lysosomes, we used time-lapse microscopy of transfected NRK cells to follow the movement of lysosomes

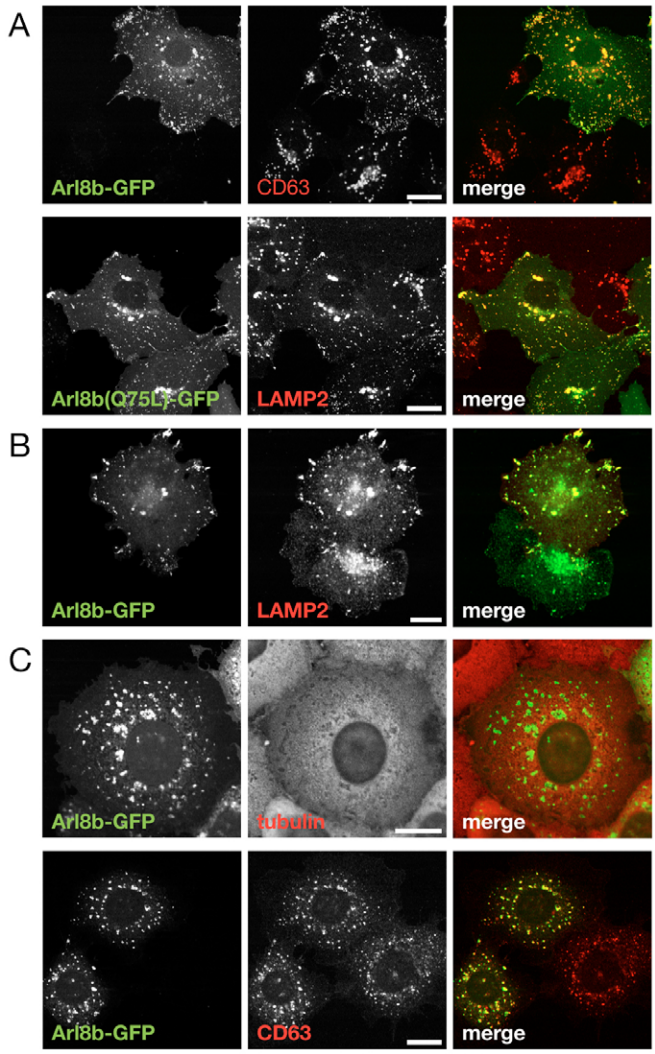
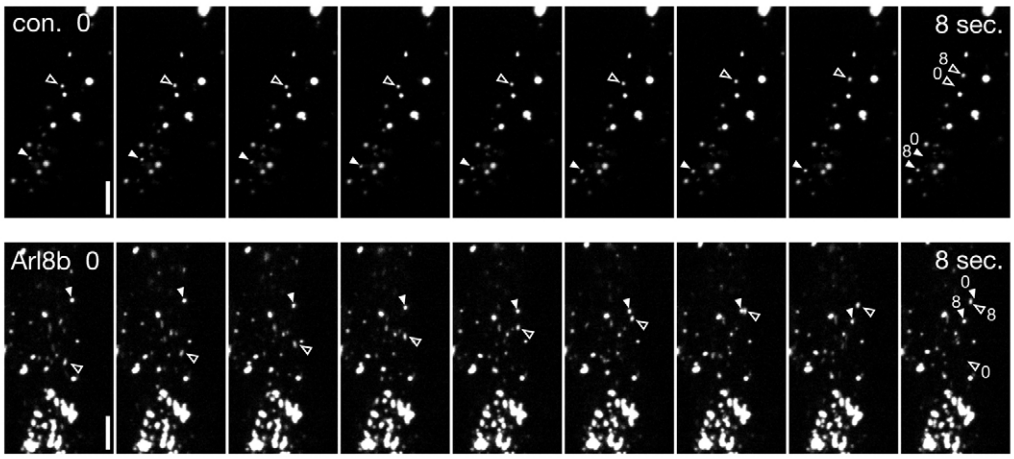


Fig. 5. Arl8b expression results in a more peripheral localization of lysosomes. (A) Confocal micrographs of COS cells expressing Arl8b-GFP or Arl8b(Q75L)-GFP and labelled with the antibodies to the indicated lysosomal membrane proteins. (B) As in (A), except that cells were trypsinised and plated on glass slides 90 minutes prior to fixation. (C) As in (A), except that cells were treated with 20 μM nocodazole for 4 hours before fixation, and then labelled for tubulin to confirm microtubule depolymerisation, or for the lysosomal antigen CD63. In such cells the lysosomes are scattered throughout the cytosol irrespective of the presence of Arl8b-GFP. Bars, 10 μm.

labelled with endocytosed dextran. As observed for fixed cells, lysosomes clustered in the cell centre, and in Arl8b-expressing cells they also accumulated at the cell periphery (supplementary material Movie 1). Although most of these lysosomes were relatively static, the movies revealed that in both transfected and untransfected cells there were, as expected, mobile lysosomes in the cytosol between the cell centre and the periphery (Matteoni and Kreis, 1987). To analyse this movement more closely, we imaged cell extensions because these allowed clear distinction between movements towards or away from the cell body. In both control and Arl8b-GFP-transfected cells, lysosomes in such extensions showed periods of relative immobility and then made sustained movements towards or away from the periphery (Fig. 6A).

A



B

	control	Arl8b	Arl8b(Q75L)
Total number of lysosomes tracked (5 cells)	521	893	975
Velocity (displacement between frames, nm/sec)	53.5 +/- 3.6	85.3 +/- 4.0	86.3 +/- 3.6
Duration of static episodes (sec)	8.58 +/- 0.86	3.74 +/- 0.28	4.01 +/- 0.45
% of lysosomes with long episodes of continuous fast motion (in 3 min)	8.1 %	16.3 %	19.1 %
Proportion of transport events toward cell body	0.49 +/- 0.02	0.50 +/- 0.01	0.52+/- 0.01
Proportion of transport events toward cell periphery	0.51 +/- 0.02	0.50 +/- 0.01	0.48 +/- 0.01
Length of episodes of continuous motion - toward cell body (nm)	316 +/- 29	425 +/- 22	456 +/- 27
Length of episodes of continuous motion - toward cell periphery (nm)	295 +/- 18	404 +/- 22	384 +/- 16

Fig. 6. Arl8b stimulates lysosomal transport. (A) Still images of spinning-disc movies of lysosomes in processes of an untransfected NRK cell (con.), or of the same expressing Arl8b-GFP (Arl8b). Lysosomes were labelled by incubating cells for four hours with 1 mg/ml Alexa-Fluor-568-dextran, followed by a 20-hour chase. In both cases the cell body is at the top, and lysosomes moving toward, or away from, the cell body are indicated with open or closed triangles respectively. Bars, 5 μm. (B) Table summarizing the analysis of movies of NRK cells transfected with Arl8b-GFP, Arl8b(Q75L)-GFP or of untransfected cells. Lysosomes were labelled as in (A) and cell extensions of five cells each were imaged for three minutes at two frames per second. Individual lysosomes were tracked relative to an axis running from the cell body along the extension, and various parameters of the movement are shown. In each case a mean value is given, along with the standard error of mean. A 'displacement' is defined as the movement between successive frames. A 'transport event' is defined as a displacement of 0.15 μm or more per second, because this is typically the minimum speed of microtubule-dependent transport in vivo (Gross et al., 2000). A 'static episode' is a period where the displacement between each successive frame does not exceed 0.15 μm/second. A 'long episode of continuous fast motion' is when the net direct displacement over eight successive frames exceeds 1 μm. The length of an 'episode of continuous motion' is defined for objects that have made a transport event, and is the net direct displacement before the object reverses direction relative to the axis, or does not make a transport event (i.e. moves less than 0.15 μm/second between frames). Perinuclear clusters of lysosomes were not included in the analysis as the software could not recognise every lysosome within these clusters.

However, in cells expressing Arl8b-GFP the lysosomes appeared to move more frequently and over greater distances (supplementary material Movies 2 and 3). This difference was confirmed by using tracking software to quantify lysosomal movements (Fig. 6B). It seemed that the major effect of Arl8b-GFP was to increase the proportion of lysosomes that showed episodes of sustained rapid motion (from 8.1% to 16.2% of lysosomes in the 3-minute imaging periods), and to increase the average displacement per frame for all movement (53.5 nm/second to 85.3 nm/ second). In addition, the average length of pauses or static episodes was reduced (from 8.6 to 3.7 seconds). The increase in movement was not directional, because the relative frequency of movements towards the periphery compared with those towards the centre was unchanged (Fig. 6B). Similar values were obtained for both Arl8b and Arl8b(Q75L), suggesting that transfection of Arl8b has a stimulatory effect due to an increase in the amount of the GTP-bound form (Fig. 6B).

By moving more frequently and over longer distances, lysosomes of Arl8b-transfected cells might have a higher chance of reaching the cell periphery where they can then either fall off the microtubules or become aggregated. The bi-directional effect on movement would explain why not all of the lysosomes accumulate at the periphery; and indeed, lysosomes can become detached from peripheral clusters and return to the cell centre (supplementary material Movie 3).

Arl8b remains associated with lysosomes during mitosis

While our analysis of Arl8a and Arl8b was in progress, Katada and co-workers described Arl8a and Arl8b as Gie1 and Gie2, GTPases that had a perinuclear localization in interphase cells, but were associated with the spindle mid-zone and the midbody in mitotic cells (Okai et al., 2004). This appears to be contrary to the lysosomal localisation observed by us. However, during mitosis many membrane traffic processes cease, and some organelles become fragmented to aid segregation of daughter cells (Shorter and Warren, 2002; Warren and Wickner, 1996). Indeed, it has been reported that Arf1 dissociates from the Golgi during mitosis (Altan-Bonnet et al., 2003), and so it was possible that Arl8 is relocalised during these mitotic processes. However, examination of Arl8b-GFP in both COS and NRK cells showed that the protein remained associated with lysosomes during mitosis and did not localise to either the spindle or midbody (Fig. 7A,B). This suggests that lysosomal targeting is not under cell-cycle regulation and that the protein is not involved in the mitotic spindle.

Drosophila Arl8 is targeted to lysosomes

The above analysis of mammalian Arl8a and Arl8b relies on tagged forms of the protein. To provide evidence that these results are likely to reflect the properties of the native proteins, we raised an antiserum against the single homologue present in *Drosophila*. This homologue is encoded by gene *CG7891* and we will refer to it as *Drosophila* Arl8. Fig. 8A shows that, when this antiserum was used to probe extracts of cultured *Drosophila* cells, it recognised a prominent band of approximately 23 kD (which is close to the predicted molecular weight of 21.3 kD) that was absent when the cells had been treated with dsRNA to silence expression of *Drosophila* Arl8. The antiserum also recognised a second prominent band, but this was not affected by Arl8 dsRNA. When used for immunofluorescence of cultured cells, the antiserum labelled punctate structures in the cytoplasm but this staining was absent following Arl8 dsRNA treatment (Fig. 8B). This indicates that, endogenous Arl8 is present in punctate cytoplasmic structures and the second band is a cross-reactivity with a distinct protein that is only recognised when denatured. The Arl8-positive structures colocalise with a GFP-fusion protein of *Drosophila* LAMP1, confirming that they are lysosomes (Fig. 8C). As with the epitope-tagged protein in mammalian cells, incubation with the anti-Arl8 antiserum did not result in any detectable labelling in the spindle mid-zone (Fig. 8D) or the midbody (Fig. 8E). Finally, the antiserum allowed detection of native *Drosophila* Arl8 expressed in COS cells, where it was seen to localise to lysosomes and also to induce the same scattering of lysosomes seen with the tagged mammalian proteins (Fig. 8F). Taken together, these results indicate that the results with the tagged forms of Arl8a and Arl8b probably reflect the properties of the native proteins.

Discussion

In this research, we have examined two closely related human Arf-like GTPases, Arl8a and Arl8b, and found that both are localized to lysosomes and, when overexpressed cause an accumulation of lysosomes at the cell periphery. In addition, we show for Arl8b that membrane targeting depends on the N-terminus, which is itself acetylated, and that lysosomal motility is stimulated by overexpression. Given that Arl8a is also a potential substrate for NatC acetylation rather than

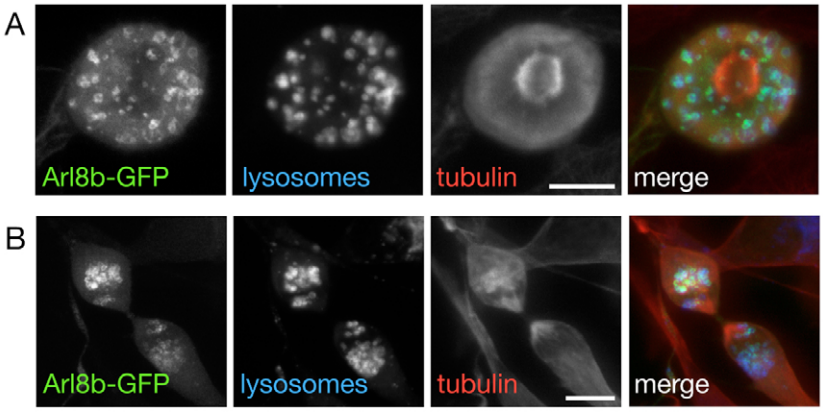


Fig. 7. Localisation of Arl8b in mitotic cells. Projection images of a stack of confocal slices from NRK cells transfected with a plasmid expressing Arl8b-GFP. After fixation and permeabilisation the cells were stained with antibodies to lysosomes and tubulin. Mitotic cells were identified in the total population by tubulin staining. Arl8b-GFP is localised to lysosomes, and is not detectable on the spindle mid-zone (A) or the midbody (B). Bars, 10 μm.

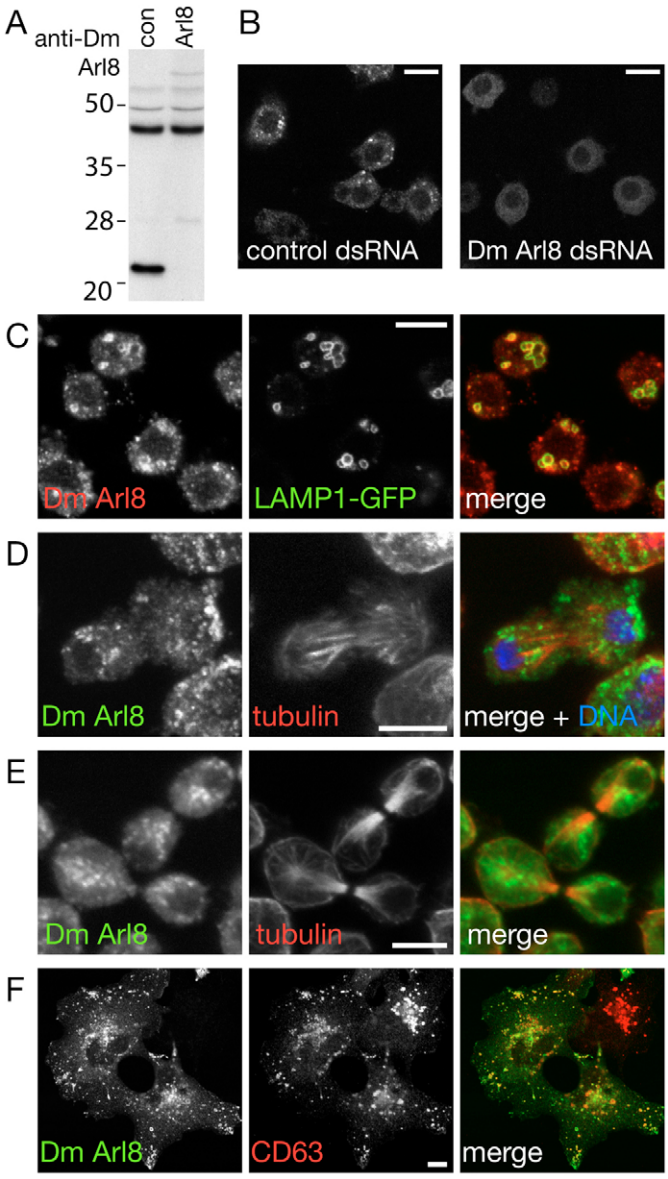


Fig. 8. Endogenous Arl8 is present on lysosomes in *Drosophila* cells. (A) Protein blot of total cellular proteins from D.Mel-2 cells probed with a rabbit antiserum raised against *Drosophila* Arl8 (Dm Arl8, CG7891). The cells were treated with dsRNA from GFP (con) or *Drosophila* Arl8. (B) Confocal micrographs of D.Mel-2 cells treated as in (A) with dsRNA from GFP (control) or *Drosophila* Arl8. After fixation and permeabilisation, cells were labelled with anti-*Drosophila* Arl8, and imaged with identical settings. (C) Confocal micrographs of S2 cells expressing a GFP fusion to the C-terminus of *Drosophila* LAMP1 (CG3305) and labelled with anti-*Drosophila* Arl8. (D-E) Confocal projection stacks of mitotic D.Mel-2 cells labelled with antibodies against *Drosophila* Arl8 and tubulin. Arl8 does not detectably concentrate in the spindle mid-zone in late anaphase (D), or in the midbodies (E) during late telophase. (F) Confocal micrographs of COS cells transfected with a plasmid expressing a native (i.e. un-tagged) form of *Drosophila* Arl8, and labelled with antibodies to *Drosophila* Arl8 and CD63. The *Drosophila* protein is localised to lysosomes, and alters the perinuclear distribution of lysosomes seen in the adjacent untransfected cell. Bars, 5 μm.

myristoylation and causes a similar alteration in lysosomal distribution when overexpressed, then it probably shares the aforementioned features with Arl8b. Both Arl8a and Arl8b have homologues in vertebrates whose genomes have been sequenced, and there is a single Arl8 homologue in many other eukaryotes including filamentous fungi, plants and protozoa, such as *Dictyostelium* (Li et al., 2004; Pasqualato et al., 2002). As such, the protein seems likely to play a role in a fundamental cellular process that is widely conserved in eukaryotic cells, and at least the *Drosophila* Arl8 homologue is also present on lysosomes. To date, Arl8a and Arl8b are the first GTPases found to be localised to lysosomes. All other organelles are known to have one or more members of the Arf or Rab families that are specifically found on their membranes, and these proteins typically serve to recruit multiple effectors that act in an organelle-specific function (Behnia and Munro, 2005; Zerial and McBride, 2001). At present, the effectors for Arl8a and Arl8b remain unknown, but overexpression of either protein causes some lysosomes to accumulate at the cell periphery. This redistribution might simply reflect the longer and more rapid movement on microtubules, resulting in some lysosomes falling off the plus-ends at the periphery and remaining there for a while before re-engaging with microtubules. However, in melanocytes a peripheral pool of melanosomes (a lysosome related organelle) is maintained by being anchored to cortical actin (Rodionov et al., 2003; Seabra and Coudrier, 2004). We cannot exclude a contribution from such actin-based anchoring, but the loss of the peripheral pool after microtubule depolymerisation implies that, if it does occur it is not sufficient by itself to confer the peripheral location.

Lysosomes, like many organelles, are capable of bi-directional movement, and this may be important to ensure efficient exposure to the whole volume of the cytoplasm. An increase in microtubule-dependent motility implies that Arl8b is capable of recruiting or activating either motor regulators, such as lipid or protein kinases, or components of the transport machinery, such as dynactin, which can recruit both plus-end and minus-end directed motors (Jordens et al., 2001; Mallik and Gross, 2004; Rodionov et al., 2003). In the long term, identification of the effectors for Arl8 will be required to understand the molecular basis of its effects. However, it is interesting that, although Arl8 is very well conserved in evolution, it has been lost from budding yeasts – which are unusual, in that all known organelle movements occur on actin rather than microtubules (Pruyne et al., 2004). Thus, understanding the role of Arl8 could be of relevance to microtubule-based motility in many systems.

Although the binding partners of Arl8a and Arl8b are still unknown, it is clear that their recruitment to lysosomal membranes depends on both the N-terminal amphipathic helix and the precise structure of the first two residues. The amphipathic helix is important for membrane binding of all members of the Arf family so far investigated, but in most cases it is preceded by an N-terminal myristoyl group that initiates and stabilises membrane association (Antonny et al., 1997; Beraud-Dufour et al., 1999). However, we find that Arl8b is N-terminally acetylated, consistent with it having a hydrophobic residue rather than a glycine in the second position. The Golgi-localised Arl ARFRP1 also has a well-

conserved hydrophobic residue in the second position, and the yeast homologue (Arl3) has been shown to be acetylated. This acetylation is necessary for the GTPase to be targeted to the Golgi by binding Sys1, a small polytopic membrane protein (Behnia et al., 2004; Setty et al., 2004). Thus N-terminal acetylation of an amphipathic helix appears to be an alternative to fatty acid modification as a targeting mechanism for small GTPases, and our results with Arl8b indicate that this alternative targeting strategy is more widely used than just ARFRP1/Arl3. However, this raises the question of how the acetylated N-termini of Arl8a and Arl8b contribute to targeting the proteins to lysosomes. Our database searches have not revealed a family of Sys1-related proteins, suggesting that any protein receptor for Arl8a and Arl8b would have to be distantly related at best. Moreover, chimeras of Arl8b and Arf1 indicated that the acetylated N-terminal amphipathic helix is neither necessary nor sufficient for lysosomal targeting. Thus, the acetylated N-terminus might not be specifically recognised by a protein receptor. Instead, the acetylation might be important for removing the positive charge of the α-amino group and stabilizing the amphipathic helix, hence, promoting its insertion into the lipid bilayer after activation by a lysosomal GEF. The putative amphipathic helices of Arl8a and Arl8b are four residues longer than those of the myristoylated Arfs Arf1 or Arl1 (Fig. 3B), and these extra hydrophobic residues might compensate for the reduction in membrane affinity due to the absence of the myristoyl group.

Arl8a and Arl8b were recently reported to localize to the mitotic spindle and the midbody during cytokinesis, with a 'perinuclear' localisation in interphase cells (Okai et al., 2004). However, we did not observe relocation of Arl8b-GFP from lysosomes to these structures in dividing cells. C-terminal tags have been used successfully with other members of the Arf family (Aikawa and Martin, 2003; Altan-Bonnet et al., 2003; daSilva et al., 2004; Vasudevan et al., 1998), and the structure of Arl8b indicates that its C-terminus should be as well-exposed as these other proteins (Protein Data Bank entry 2AL7) but, nonetheless, it is possible that the C-terminal GFP tag used in our studies blocks the targeting of Arl8b to these mitotic structures. However, an antiserum against *Drosophila* Arl8 detected the endogenous protein on lysosomes but did not show any concentration of labelling on the spindle or midbody, although we cannot, of course, exclude the possibility that a small fraction of Arl8 is on the spindle. Some of the localisation data by Okai et al. were based on Arl8b with an N-terminal FLAG tag (Kurosu and Katada, 2001; Okai et al., 2004), and in the light of our results on the importance of the N-terminus, this N-terminally tagged protein was unlikely to be localised correctly. However, the remainder of the data was obtained with an antibody to the endogenous protein, although the specificity of this antibody was not confirmed by RNA interference.

Thus, Arl8a and Arl8b are probably on lysosomes in interphase and mitotic cells, and although they can potentially have a role on mitotic structures this might well bare further examination. Given the importance of GTPases in the functions of the organelles on which they are found, Arl8a and Arl8b appear good candidates to play a role in at least some of the processes that depend on lysosomes, such as autophagy, receptor downregulation, phagocytosis and plasma membrane repair.

Materials and Methods

Tissue culture and microscopy

COS and NRK cells were transfected with FuGene (Roche), split onto glass slides, fixed 24-40 hours after transfection with 4% paraformaldehyde, permeabilised with 0.5% Triton X-100 in PBS, blocked with 20% foetal calf serum supplemented with 0.5% Tween 20 in PBS, and labelled with antibodies in the same solution. *Drosophila* D.Mel-2 cells (Invitrogen) were grown in GIBCO *Drosophila* SFM (Invitrogen), and fixed and processed for immuno-labelling as above. Double-stranded RNA (T7 RiboMAX, Promega), was used for gene silencing as described previously (Bettencourt-Dias et al., 2005). A *Drosophila* S2 cell line stably transfected with *Drosophila* LAMP1-GFP (CG3305) under the control of a metallothionein promoter was kindly provided by Gudrun Ihrke and grown in Schneider's medium supplemented with 10% foetal calf serum. LAMP1-GFP expression was induced by treatment for three hours with 1 mM $CuSO_4$, followed by incubation in $CuSO_4$-free medium for 1-2 days.

A rabbit antiserum against residues 18-186 of *Drosophila* Arl8 (CG7891) expressed as a GST fusion from vector pGEX-6P-2 (Amersham Bioscience), was raised commercially (Harlan Sera-Lab) and affinity purified. Other rabbit antibodies were against mannose-6-phosphate receptor, giantin (Seelig et al., 1994) and the HA-epitope tag (Santa Cruz). Mouse monoclonals antibodies were to against CD63 (Fraile-Ramos et al., 2001), EEA1 and golgin-245, LAMP2 (H4B4, DSHB, Iowa), and tubulin (Sigma). Primary antibodies were detected with Alexa-Fluor-labelled secondary antibodies (Molecular Probes). Cells were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc.), and images obtained on a Radiance confocal microscope (BioRad).

Live cell imaging was performed on a spinning disc microscope (UltraView, Perkin Elmer). Cells expressing GFP-fusions were identified, and dextran-labelled lysosomes were imaged at 30°C in DMEM supplemented with 10 mM HEPES pH 7.2. Lysosomes in movies were tracked and analysed using Mathematica 4.0 (Wolfram Research) running custom software (Bullock et al., 2003).

Analysis of Arl8b by mass spectrometry

COS cells expressing Arl8b-HA were lysed 48 hours post transfection by exposure to 1 ml lysis buffer for 10 minutes on ice [50 mM Tris HCl pH 8, 150 mM KCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors (Roche)] followed by scraping. The lysate was centrifuged at 10,000 ***g*** for 10 minutes at 4°C, and the supernatant incubated for 2 hours at 4°C with 45 μl of anti-HA beads (Santa Cruz). After four washes in lysis buffer, bound protein was eluted with 100 μl of 0.5 M acetic acid pH 3.4, lyophilised and resuspended in SDS sample buffer. Following gel electrophoresis and staining with Coomassie blue, the protein was excised and digested with trypsin (Roche). Peptides were analysed using MALDI mass spectrometry (Voyager-DE, PerSeptive Biosystems) (Shevchenko et al., 1996), or sequenced with tandem mass spectrometry (QStar, Applied Biosystems).

Palmitate labelling

COS cells in 6-well plates expressing Arl8b-HA as above were labelled 40 hours post-transfection by incubation in labelling medium (DMEM, 10% dialyzed FCS) for 1 hour, followed by 1 ml of labelling medium with 0.5 mCi [^{3}H]palmitate (Amersham Biosciences) for 3 hours. Cells were washed three times with cold PBS, scraped into 500 μl of lysis buffer, incubated for 10 minutes at 4°C, and centrifuged for 10 minutes at 10,000 ***g***. The supernatant was incubated with 20 μl anti-HA beads (Santa Cruz) for 2.5 hours at 4°C, beads were washed three times with cold lysis buffer and eluted with SDS sample buffer containing 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as reducing agent. Following gel electrophoresis, and incubation in Amplify (Amersham), the gel was dried and imaged by fluorography. A proportion of the precipitate was also analysed by protein blotting with rabbit anti-HA antibody, detected with horseradish peroxidase (HRP)-conjugated anti-rabbit Ig and enhanced chemiluminescence (Amersham Biosciences).

We are greatly indebted to Simon Bullock for help with spinning-disc microscopy and analysis of lysosome movements. We also thank Farida Begum and Sew Peak-Chew for mass-spectrometry, Oliver Daumke for help with HPLC, Gudrun Ihrke for the LAMP1-GFP cell line and Matthew Freeman, Alison Gillingham and Katja Röper for comments on the manuscript. I.H. was supported by a PhD Scholarship from the Boehringer Ingelheim Fonds.

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