The Menkes disease ATPase (ATP7A) is internalized via a Rac1-regulated, clathrin- and caveolae-independent pathway

Christian Cobbold1, Julie Coventry1, Sreenivasan Ponnambalam2 and Anthony P. Monaco1,*

1Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Headington, Oxford OX3 7BN, UK and 2School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK

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The Menkes disease gene encodes a P-type transmembrane ATPase (ATP7A) that translocates cytosolic copper ions across intracellular membranes of compartments along the secretory pathway. ATP7A moves from the trans-Golgi network (TGN) to the cell surface in response to exogenously added copper ions and recycles back to the TGN upon copper removal. The protein contains a C-terminal di-leucine motif necessary for internalization from the cell surface. In this study we show that ATP7A is internalized by a novel pathway that is independent of clathrin-mediated endocytosis. Expression of dominant-negative mutants of the dynamin-I, dynamin-II and Eps15 proteins that block clathrin-dependent endocytosis of the transferrin receptor do not inhibit internalization of endogenous ATP7A, or an ATP7A reporter molecule (CD8-MCF1). Similarly, inhibitors of caveolae-mediated uptake do not affect ATP7A internalization whilst preventing uptake of PODIPY-ganglioside GM1, a caveola marker. In contrast, expression of a constitutively active mutant of the Rac1 GTPase inhibits plasma membrane internalization of both the ATP7A and transferrin receptor transmembrane proteins. These findings define a novel route required for ATP7A internalization and delivery to endosomes.

INTRODUCTION

Menkes disease is a fatal neurodegenerative disorder of copper, which leads to death in early childhood (1). The disease results from mutations in the ATP7A (ATP7A) gene, which encodes a P-type copper-transporting ATPase (2,3). Studies have localized ATP7A to the trans-Golgi network (TGN) (4–8), where it pumps copper across the TGN membrane to copper-requiring proteins and enzymes (9). Elevation in extracellular copper ion levels results in Cdc42 and protein kinase A-regulated trafficking of ATP7A to the cell surface (7), where it translocates copper ions from the cytosol to the extracellular milieu (5,10). Redistribution of the ATP7A protein from the plasma membrane to the TGN occurs when copper levels in the cytosol return to physiological levels.

The Menkes disease protein contains eight transmembrane domains, and a specific transmembrane domain has been shown to be essential for TGN localization of ATP7A (11). A second localization signal in the ATP7A protein is a di-leucine motif that regulates internalization at the plasma membrane and mediates endosomal targeting (12,13). Many proteins with di-leucine motifs are efficiently internalized via clathrin-coated pits and vesicles (14,15). However, several studies have established the existence of clathrin-independent internalization routes, for example, specialized cholesterol-rich domains on the cell surface, called caveolae, are involved in the internalization of certain proteins, viruses and toxins (16,17). Elucidation of the mechanisms regulating clathrin-mediated endocytosis has been dependent on the identification and characterization of factors needed in specific steps along this route (18). For example, the Eps15 polypeptide interacts with several proteins that associate with clathrin-coated vesicles, including the AP2 plasma membrane adaptor complex (19). Dominant negative dynamin-1, the neuronal isoform of dynamin, has been widely used to study internalization (20), and regulates the scission of endocytic vesicles from the plasma membrane.

Overexpression of dominant-negative mutants (K44A) of dynamin-I (21,22), dynamin-II, the ubiquitous dynamin isoform (23), and Eps15 (24) inhibit clathrin-mediated endocytosis (25,26). Interestingly, K44A dynamin also inhibits uptake
through caveolae (16,21,27). Specific compounds can inhibit uptake of molecules via caveolae and lipid rafts (17,28) and can be used to discriminate between these two plasma membrane internalization routes.

More recently, a role for the Rho GTPase proteins such as Cdc42, Rac1 and RhoA has been implicated in endocytosis (29,30). Rho GTPases primarily regulate dynamics of the actin cytoskeleton and directly or indirectly regulate plasma membrane-based internalization (31). Activated Rac1 and RhoA inhibit transferrin receptor-mediated endocytosis through clathrin-coated pits (29). In contrast, dominant-negative Cdc42 prevents internalization of GPI-anchored proteins by a mechanism that is clathrin- and caveolae-independent (30). Many studies have concentrated on the identification of downstream targets of these GTPases. Putative effectors that are activated by Rac1 include WAVE/Scar, POR-1 (partner of Rac) and JNK (Jun kinase), whilst Rho-associated kinase and Dia 1/2 (Diaphanous-related) can be activated by RhoA (32).

A key question is the mechanism by which multiple transmembrane domain proteins such as ATP7A are internalized in eukaryotes. To address this issue we have examined early events in the internalization of endogenously expressed full-length human ATP7A, and compared this to internalization of a single transmembrane ATP7A reporter molecule (CD8-MCF1). By overexpressing key regulators of receptor-mediated endocytosis and actin dynamics and using specific chemical inhibitors on cultured cell lines, evidence is presented for the existence of a novel pathway for targeting the ATP7A transporter to the endosome-lysosome network.

RESULTS

ATP7A is internalized from the plasma membrane via a clathrin-independent process

Previous work has suggested that ATP7A cycles constitutively between the TGN and plasma membrane (4,5). The discovery of a cytosolic, carboxy-terminal di-leucine motif in ATP7A that could confer internalization of both a reporter protein and the full length M enkes protein (12,13) indicates that this transmembrane protein could be transported via clathrin-coated pits and vesicles. Dynamin I, dynamin II and Eps15 are key proteins that regulate the scission of clathrin-coated pits to form vesicles (23,33). Expression of the GTPase-defective (K44A) dynamin-I mutant (22), dynamin-II mutant or the Eps15-EK29 mutant in transfected cells inhibits clathrin-mediated endocytosis (25,26). If ATP7A is internalized by clathrin-mediated endocytosis then it should accumulate at the plasma membrane of cells where this process has been blocked. To test this idea we overexpressed dominant-negative mutant proteins of these key regulators in transiently transfected HeLa cells and tested localization of ATP7A and the transferrin receptor, TfR (Fig. 1). ATP7A Golgi localization (Fig. 1A–C; red) is unaffected in cells expressing the dominant-negative K44A dynamin-I mutant (Fig. 1A; green), K44A dynamin-II mutant protein (Fig. 1B; green), or the Eps15 mutant (Fig. 1C; green). The TfR is internalized via clathrin-coated vesicles from the cell surface to endosomes (20). To confirm that endocytosis via clathrin was inhibited in our system, the internalization of TfR was analysed in cells expressing K44A dynamin-I, -II or Eps15-EK29. TfR endocytosis was inhibited in HeLa cells expressing K44A dynamin-I (Fig. 1D), K44A dynamin-II (Fig. 1E) and Eps15-EK29 (Fig. 1F); these results on TfR endocytosis are similar to previous studies by other groups.

One possibility is that under steady-state conditions ATP7A does not appreciably recycle between the TGN and the plasma membrane. Thus, expression of dominant-negative endocytic regulators may not affect ATP7A Golgi localization and any such effects may only be evident if a larger proportion of ATP7A was present at the plasma membrane in the first instance. Elevation of extracellular levels of copper ions (see Materials and Methods) causes translocation of ATP7A with a several-fold elevation in plasma membrane ATP7A levels (4,7,34,35); copper washout causes a return to the steady-state localization. To determine the time course of ATP7A internalization from the cell surface, cells were incubated with copper for 2 h to allow ATP7A to accumulate at the cell surface, and then processed for immunofluorescence microscopy at increasing times after copper removal. Quantitative analysis (see Materials and Methods) shows that very little ATP7A was internalized at early time points following copper removal, however, uptake was essentially complete within 6 h (Fig. 2A).

We next tested copper level elevation and washout in HeLa cells expressing the dominant-negative K44A dynamin-I mutant, K44A dynamin-II mutant, or the Eps15 mutant protein. Incubation with elevated levels of copper ions (200 μM) in cells expressing K44A dynamin-I, -II or Eps15-EK29 show strong ATP7A staining at the cell surface (Fig. 2B–D). Copper was removed from cells and internalization allowed to proceed for 6 h, the time taken for ATP7A to internalize under control conditions (Fig. 2A). In cells expressing K44A dynamin-I (Fig. 2E; green), -II (Fig. 2F; green), or Eps15-EK29 (Fig. 2G; green), only a juxtanuclear Golgi-like localization is observed for the ATP7A protein with little plasma membrane staining now detected (Fig. 2E–G; red). This shows that ATP7A is efficiently internalized in cells defective in clathrin-mediated endocytosis. In control copper washout experiments, the internalization of the transferrin receptor was inhibited in K44A dynamin-I, -II and Eps15-EK29 transfected HeLa cells during the 6 h time course (Fig. 2H–J). Significantly, expression of dynamin-I, -II or Eps15-EK29 at high or relatively low levels inhibited TfR endocytosis, whilst having no effect on ATP7A internalization.

The carboxy-proximal 93 amino acids of ATP7A are sufficient to confer di-leucine mediated internalization of a reporter molecule (12). To define a role for clathrin-mediated internalization in this process, the CD8-MCF1 hybrid protein (12) was expressed in transfected COS-1 cells (Fig. 3). Incubation of cells with exogenously added anti-CD8 antibodies prior to internalization allowed us to monitor uptake of this hybrid fusion protein. Cells expressing this hybrid protein under steady-state conditions showed a punctate, juxtanuclear and peripheral staining pattern that is characteristic of early and late endosomes (12) (Fig. 3A). Cell surface-bound antibody is also transported to this compartment in transfected cells (Fig. 3B). Expression of the K44A dynamin-I mutant GTPase (Fig. 3C) or K44A dynamin-II mutant (Fig. 3E) do not affect CD8-MCF1 internalization (Fig. 3D and F, respectively;
arrowheads). Similarly, expression of Eps15-EK29 mutant (Fig. 3G) does not significantly affect CD8-MCF1 internalization (Fig. 3H). In contrast, control COS-1 cells expressing K44A dynamin-I, -II or Eps15-EK29 proteins showed substantial inhibition of labeled transferrin uptake (data not shown).

ATP7A is internalized from the plasma membrane via a caveolae-independent process

The above observations suggested that ATP7A is internalized through a clathrin-independent route. One possibility is that ATP7A is internalized from the plasma membrane via caveolae or lipid rafts. However, K44A dynamin-I and -II inhibit uptake of molecules and certain animal viruses through caveolae (16,21,27), suggesting that ATP7A does not use this pathway. To confirm this, chemicals that disrupt caveolae or lipid rafts were added to cells during the copper treatment and washout assay. We performed copper washout and microscopy-based internalization assays of ATP7A from the cell surface in the presence of filipin, methyl-β-cyclodextrin or nystatin for 30 min at 37°C. The OKT8 antibody that binds to an extracellular epitope in CD8-MCF1 was bound at 4°C. Cells were then transferred to 37°C for 30 min in the presence of a caveolae/lipid raft chemical inhibitor and internalization of CD8-MCF1 monitored. Figure 3B shows efficient internalization of the antibody/CD8-MCF1 complex under control conditions. Significantly, when the same experiment is performed in the presence of nystatin (Fig. 4E) or methyl-β-cyclodextrin (Fig. 4F), the OKT8/CD8-MCF1 complex was efficiently internalized and shows a punctate, juxtanuclear and peripheral staining pattern characteristic of early and late endosomes. The same experiment with filipin showed similar results (data not shown).

It has recently been demonstrated that glycosphingolipids are internalized from the plasma membrane by a clathrin-independent, caveolar-related mechanism (36,37). To confirm that the chemicals used in this study were inhibiting caveolae-mediated uptake in HeLa cells, the internalization of the fluorescent glycosphingolipid, BODIPY-C₅-ganglioside GM₁ (BODIPY-GM₁), was analysed. Cells were incubated in the absence or presence of methyl-β-cyclodextrin and nystatin for 30 min at 37°C. Under control conditions BODIPY-GM₁ was internalized into the cytoplasm (Fig. 5A); however, when cells were incubated in the presence of methyl-β-cyclodextrin, no internalization could be detected (Fig. 5B). Similarly, nystatin inhibited internalization of BODIPY-GM₁ from the cell surface (Fig. 5C). These data show that the chemicals used were efficiently inhibiting protein uptake through caveolae.
Figure 2. ATP7A internalization is independent of clathrin. (A) Time course of ATP7A internalization. HeLa cells treated with 200 μM copper chloride for 2 h, followed by washout and recovery with cycloheximide in the absence of copper for increasing times were processed for immunofluorescence analysis. The histogram shows the quantification of the relative levels of plasma membrane ATP7A following copper washout and recovery (n = 20 for each). (B–G) ATP7A, but not TfR, internalizes independently of clathrin. Transfected HeLa cells expressing K44A dynamin-I (B, E, H), K44A dynamin-II (C, F, I), or Eps15-EK29 (D, G, J) treated with 200 μM copper chloride (B–D) followed by washout and recovery with cycloheximide (E–G) were processed for immunofluorescence analysis using mouse anti-HA (green; B, E, H), mouse anti-myc (green; C, F, I), or rabbit anti-ATP7A (red; B–G). Internalized rhodamine-conjugated transferrin for 6 h (red; H–J). Eps15-EK29 was detected through its GFP tag.
ATP7A is internalized from the cell surface via a Rac1-dependent process

The regulation by different Rho GTPase proteins of actin cytoskeleton dynamics is now well established (38). A role for small Rho GTPases in trafficking of proteins through both the secretory and endocytic pathways has recently been highlighted.

Figure 3. CD8-MCF1 internalization is independent of clathrin. Transfected COS-1 cells expressing CD8-MCF1 (A–H), K44A-dynamin-I (C), K44A-dynamin-II (E) and Eps15-EK29 (G) were allowed to bind anti-CD8 antibodies before internalization and processing for immunofluorescence analysis. (A) A conventional confocal analysis of the steady-state localization of the CD8-MCF1 protein for comparison, using mouse anti-CD8 followed by FITC-conjugated anti-mouse. Bound mouse anti-CD8, i.e., OKT8 (B, D, F, H) and mouse anti-HA (C) were detected using Texas Red-conjugated anti-mouse IgG2a and FITC-conjugated anti-mouse IgG2b secondary antibodies, respectively. Dynamin-II (E) was detected using rabbit anti-myc followed by FITC-conjugated anti-rabbit secondary antibody. Eps15-EK29 was detected through the GFP tag.

Figure 4. ATP7A is internalized from the plasma membrane via a caveolae-independent process. HeLa cells treated with 200 μM copper chloride (A) followed by washout and recovery with cycloheximide in the absence (B), or presence of nystatin (C) or methyl-β-cyclodextrin (D), and processed for immunofluorescence analysis. Bound rabbit anti-ATP7A was detected using Texas Red-conjugated anti-rabbit secondary antibody. Transfected COS-1 cells expressing CD8-MCF1 (E, F) were incubated with nystatin (E) or methyl-β-cyclodextrin (F) for 30 min at 37°C. Cells were allowed to bind anti-CD8 antibodies before internalization in the presence of nystatin (E) or methyl-β-cyclodextrin (F), and processed for immunofluorescence analysis. Bound mouse anti-CD8, i.e., OKT8, was detected using FITC-conjugated anti-mouse IgG2b secondary antibody. Histogram showing quantification of the relative levels of plasma membrane ATP7A following copper washout and recovery (n = 30 for each).
(7,29,30). As our previous results (Figs 1–4) show that ATP7A is not internalized via a clathrin- or caveolae-dependent route, there was a possibility that one or more Rho GTPases regulate ATP7A internalization. One approach that has addressed this is overexpression of dominant-negative (DN) or constitutively active (CA) mutant Rho GTPases that regulate the actin cytoskeleton (29,30,38,39).

To test a role for Rho GTPases on ATP7A internalization at the plasma membrane, elevated copper followed by copper washout and retrieval/endocytosis of ATP7A from the cell-surface was performed. HeLa cells expressing the mutant Rho GTPases were incubated with copper for 2 h to allow ATP7A to traffic to the plasma membrane. With the exception of cells expressing CA Cdc42, all cells showed strong staining for ATP7A at the cell surface (data not shown). Importantly, we have previously shown that CA Cdc42 (Cdc42V12) inhibits trafficking of ATP7A from the TGN to the cell surface of HeLa cells (7). Cells were then incubated in normal media lacking copper for 6 h. A juxtanuclear Golgi localization was observed for the ATP7A protein in cells expressing DN Cdc42 (Fig. 6A and B), DN Rac1 (Fig. 6C and D), and DN RhoA (Fig. 6E and F). In contrast, cells expressing CA Rac1 (Fig. 6G and H) showed substantial inhibition of ATP7A uptake. Significant levels of ATP7A were now observed at the cell surface (Fig. 6G). Quantitative analysis (Fig. 7E) showed that internalization was inhibited ~2.5-fold compared to controls. Overexpression of CA RhoA (Fig. 6I) inhibited ATP7A internalization to a perinuclear location (Fig. 6I); however, only a slight increase in protein levels was observed at the surface of these cells. Quantitative analysis reveals a 1.6-fold increase in cell surface ATP7A levels (Fig. 7E).

To further test a role for CA Rac1-mediated internalization of ATP7A, and also to determine the effect of CA Cdc42 on ATP7A endocytosis, the CD8-MCF1 hybrid protein (12) was expressed in transfected COS-1 cells. Incubation of cells with exogenously anti-CD8 antibodies prior to internalization allowed us to monitor uptake of this hybrid fusion protein from the cell surface. In cells expressing CA Cdc42 (Fig. 7B), the OKT8/CD8-MCF1 complex was efficiently internalized from the cell surface and showed a punctate, juxtanuclear and peripheral staining pattern characteristic of early and late endosomes (Fig. 7A), showing that Cdc42 was not involved in the internalization of ATP7A from the plasma membrane. These data also show that CD8-M CF1 is continually recycling between the cell surface and TGN/endosome network in CA Cdc42-expressing cells. However, when the same experiment was performed in cells expressing CA Rac1 (Fig. 7D), the OKT8-CD8-MCF1 complex remained at the cell surface (Fig. 7C; arrowhead), confirming our previous observation that Rac1 was involved in the internalization of ATP7A.

DISCUSSION

The Menkes disease protein is a key copper transporter containing eight transmembrane domains. In this study we used the full-length and endogenous ATP7A protein as a tool to study the internalization of a multiple transmembrane domain protein. We previously showed that ATP7A is efficiently internalized from the cell surface into the endocytic/recycling pathway through a di-leucine motif at its C-terminus (12). The C-terminal di-leucine motif is common among transmembrane proteins that internalize via clathrin-mediated endocytosis. As other groups have postulated that ATP7A continuously recycles between the TGN and cell surface (5), it is likely that ATP7A is internalized by clathrin-dependent receptor-mediated endocytosis. However, inhibition of clathrin-mediated endocytosis using dominant-negative dynamin-I, -II and Eps15, failed to alter the steady-state Golgi distribution of ATP7A. This was a surprising, although somewhat similar, result to a previous study on another TGN recycling protein, TGN46 (40).

Another possibility was that ATP7A did not significantly constitutively recycle between the TGN and cell surface under steady-state conditions; thus endocytic regulation of ATP7A uptake would be negligible or non-existent under such conditions. A copper treatment and washout assay allows us to detect a significant proportion of ATP7A that is redistributed to the plasma membrane; this plasma membrane ATP7A is then returned to the Golgi apparatus upon copper washout. Digital-based microscopy techniques allow us to quantify the proportion of ATP7A present at the plasma membrane in a statistically significant manner. In cells expressing the dominant-negative mutant dynamin-I, -II and Eps15 proteins, both ATP7A and the ATP7A reporter molecules (CD8-MCF1) are efficiently recycled back to the Golgi apparatus, thus showing that ATP7A internalization is independent of clathrin-mediated
endocytosis. However, this is in contrast to a similar study on TGN46, which showed that plasma membrane internalization was dynamin I-dependent (40). Significantly, endocytosis of the transferrin receptor was inhibited in cells expressing these mutant proteins.

As the ATP7A protein did not internalize by clathrin-mediated endocytosis it was possible that internalization occurred via caveolae or lipid rafts. However, the caveolae-disrupting agents nystatin (41), filipin and methyl-β-cyclodextrin (42) did
not inhibit internalization of plasma membrane ATP7A or CD8-M CF1 supporting the existence of a trafficking route for ATP7A that is independent of caveolae and lipid rafts. Significantly, K44A dynamin-I and -II inhibit caveolae-, as well as clathrin-mediated endocytosis (16,21,27). Thus, any ATP7A internalization obtained upon expression of either dominant-negative dynamin-I or -II is likely to reflect the amount of plasma membrane protein taken up by clathrin- and caveolae-independent endocytosis. HeLa cells have very low levels of caveolin-1 and few distinguishable caveolae on the plasma membrane (28), again arguing that this route is unlikely to play a major role in ATP7A trafficking.

It is now well established that Rho GTPases play an important role in controlling vesicular trafficking and the organization dynamics of the actin cytoskeleton (38,43). To date, the most compelling evidence of a role for Rho GTPases in endocytosis from the cell surface has come from studies on Rac1, RhoA and Cdc42 (29,30). The endocytosis of the transferrin receptor through clathrin-coated vesicles is inhibited in cells expressing activated Rac1 and RhoA. Likewise, expression of dominant negative Cdc42 inhibits the internalization of GPI-anchored proteins through a clathrin and caveolae-independent manner (30). Our studies show that activated Rac1 inhibits both the internalization of ATP7A, and the CD8-M CF1 reporter molecule, from the plasma membrane.

We also searched for the downstream targets of Rac1 that may be involved in ATP7A internalization. Rac1 has many downstream targets; it can directly activate JNK, POR1 and PAK (p21-associated kinase); PAK can regulate the Rac1-induced activation of JNK (44), whilst deletion mutants of POR1 inhibit the induction of membrane ruffling by activated Rac1 (45).

Overexpression of the double Rac1 mutants, Racv12L37 and Racv12H40, have been shown to activate JNK (but not POR1) and POR1 (but not JNK), respectively (46). We have shown that Racv12L37 inhibited the internalization of ATP7A from the plasma membrane, while Racv12H40 had little effect (C. Cobbold, unpublished observations). These findings suggest that the JNK regulator is linked to a step that controls ATP7A internalization from the cell surface. However, expression of a constitutively active form of JNK (47) did not inhibit ATP7A internalization (C. Cobbold, unpublished observations). Similarly, ATP7A internalization was not significantly inhibited when cells were treated with JNK activators (48,49) such as tumour necrosis factor α and anisomycin (C. Cobbold, unpublished observations). Thus Rac1 could activate specific downstream regulators, possibly in association with JNK, which in turn control the rate of ATP7A uptake from the plasma membrane.

The results described here call into question the role of di-leucine motifs in internalization events solely involving clathrin-mediated transport. Interestingly, in primary rat adipose cells the GLUT4 glucose transporter is internalized through a clathrin-dependent route that is independent of its C-terminal di-leucine motif (50). In this case, the GLUT4 C-terminal domain interacts with C-Daxx, an adaptor protein associated with the Fas and type II TGF-beta receptors (51), and this regulates its internalization (52). Recent data has shown that the interleukin-2 receptor (IL-2R) is internalized in a clathrin-independent process in lymphocytes (53); however these cells do not contain caveolae. Although the mechanism of IL-2R internalization in this cell type is not clear, it appears to be coupled to the IL-2R partitioning into lipid rafts. In the case of ATP7A, one possibility is that its C-terminal di-leucine motif associates with factors that control its internalization, but in a pathway that is different from the AP2/clathrin route that regulates TFR uptake. For example, yeast alkaline phosphatase is directed via an AP-3-dependent mechanism from the distal Golgi to the yeast vacuole via a di-leucine-like sorting signal (54). Although the majority of evidence suggests that AP-3 and AP-4 adaptor complexes function in Golgi-to-endosome/vacuole/lysosome transport (55), it is possible that these complexes may be involved in distinct transport events from the plasma membrane for a subset of cellular membrane proteins. Such events may not require clathrin; there is debate as to whether the AP-3 and AP-4 adaptor complexes bind clathrin in vivo (55). Thus, although most membrane proteins are internalized in clathrin-coated pits, other pathways are capable of selective, receptor-mediated endocytosis (Fig. 8). Future studies will determine the exact nature of the cytosolic and membrane-bound machinery needed to facilitate the internalization of ATP7A.

MATERIALS AND METHODS

Reagents and cell lines

All reagents and chemicals were from Sigma (Poole, UK) unless otherwise stated. BODIPY-C 5-ganglioside GM 1 was from Molecular Probes. HeLa and COS-1 cells were from ECACC (Porton Down, UK) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine supplemented with penicillin/streptomycin.

Immunofluorescence analysis and antibodies

For indirect immunofluorescence analysis, cells were grown to 40-80% confluence on glass coverslips, fixed in 4% (w/v) paraformaldehyde or –20°C methanol and processed as previously described (56). The R70 rabbit antibody has been described previously (7). Mouse monoclonal antibodies used were T7-tag to the T7 epitope (Novagen), FLAG M2 to the FLAG tag, OKT8 to human CD8α, 9E10 to human myc, and 12CA5 to the viral HA epitope tag (Sigma). Secondary antibodies used were FITC or Texas Red conjugated reagents from Molecular Probes (Eugene, OR, USA). Cells were viewed using a Nikon Optiphot microscope and a 60× oil objective operating on a Bio-Rad 1024 confocal system. Images were collected using the Bio-Rad Lasersharp software for viewing, analysis and quantitation. To avoid bleed-through from different channels, sequential capture of each image was performed on double-labeled samples. Fluorescence intensity was estimated as an average pixel intensity of digitized images measured from the plasma membrane regions of cells using laser scanning confocal microscope analysis software (Bio-Rad Laboratories). Each experiment was repeated a minimum of three times.
Transfection studies

The following constructs in mammalian expression vectors were gifts as indicated: dominant-negative mutants of dynamin-I and Eps15 were from Eamonn Kelly (University of Bristol, UK); dominant-negative mutant of dynamin-II from Harvey McMahon (MRC-LMB, Cambridge, UK); dominant-negative and constitutively active mutants of Cdc42, Rac1 and RhoA were as described (39) and provided by Romano Regazzi (University of Lausanne, Switzerland); RacV12L37 and RacV12H40 constructs previously described (46) were given by Linda Van Aelst (Cold Spring Harbor Laboratory, NY, USA), and Professor U. Rapp (Universitat Würzburg, Germany) gave the SAPK-beta-MKK7 constructs (47). Cells were treated with Genejuice transfection reagent (Novagen, UK) and 1 μg/ml DNA using the manufacturer's protocol. After 20 h treatment,
cells were washed and subsequently cultured for 24 h in DMEM containing 2% FBS before treatment as indicated in the figure legends.

Internalization assays

Approximately 24–48 h after transient transfection using Genejuice (Novagen, UK), cells were washed four times with serum-free DMEM and incubated for 15 min at 37°C with 0.5% BSA/DMEM to block non-specific antibody binding sites. Cells were then incubated with OKT8 tissue-culture supernatant diluted 1:25 in serum-free DMEM at 4°C for 30 min. Unbound antibody was removed by washing cells with serum-free DMEM before incubation for 30 min at 37°C to allow antibody internalization in the presence or absence of Texas Red-conjugated human transferrin (final concentration, 50 μg/ml; Molecular Probes, Eugene, OR, USA). Cells were then processed for immunofluorescence as described above.

The internalization of BODIPY-C5-ganglioside GM1, complexed with defatted bovine serum albumin, was essentially performed as previously described (36,37).

Copper washout and internalization assay

Untransfected HeLa cells, or cells expressing K44A dynamin-I, K44A dynamin-II, Eps15-EK 29, RacV12C37, RacV12H40 or mutant Rho GTPase proteins were treated with 200 μM copper chloride for 2 h at 37°C. Cells were washed once with phosphate-buffered saline, and then incubated with serum-free DMEM without copper in the presence of 200 μM of the copper chelator, bathocuproinedisulfonic acid, and 50 μg/ml cycloheximide, a protein synthesis inhibitor, for 6 h; the media was replaced twice. Addition of cycloheximide during copper washout ensures that internalized ATP7A is measured, and not ATP7A synthesized during the time course of the experiment. Filipin (1 μg/ml), methyl-betacyclodextrin (5 μM), and nystatin (25 μg/ml) were added to cells for 6 h as indicated in the figure legends. Cells were then processed for confocal microscopy as described above.

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