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Arf6 and microtubules in adhesion-dependent trafficking of lipid rafts

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SUMMARY

Integrin-mediated adhesion regulates Rac1 membrane binding sites within lipid rafts. Detachment of cells from the substratum triggers clearance of rafts from the plasma membrane through caveolin-dependent internalization. The small GTPase Arf6 and microtubules also regulate Rac-dependent cell spreading and migration but the mechanisms are poorly understood. We now show that endocytosis of rafts after detachment requires F-actin, followed by microtubule-dependent trafficking to recycling endosomes (RE). When cells are replated on fibronectin, rafts exit from RE in an Arf6-dependent manner and return to the plasma membrane along microtubules. Both of these steps are required for plasma membrane targeting and activation of Rac1. These data therefore define a novel membrane raft trafficking pathway that is crucial for anchorage-dependent signaling.

INTRODUCTION

The small GTPases Rac, Rho and Cdc42 localize to ordered plasma membrane microdomains called lipid rafts^{1–6}. These regions are enriched in cholesterol, sphingolipids, glycosylphosphatidylinositol (GPI)-linked and other proteins^{7, 8}. Though some aspects of raft biology have been controversial, a consensus is emerging that they are small, dynamic structures whose properties are strongly influenced by their protein content⁹. They can modulate many signaling pathways in diverse biological processes such as cell division, apoptosis, adhesion, and chemotaxis^{7, 8}. Lipid rafts modulate spatial targeting of GTPases required for cell spreading and migration^{2, 10}. Interestingly, adhesion sites have very high membrane order, suggesting a raft-like composition¹¹.

In anchorage-dependent cells, loss of integrin-mediated adhesion triggers rapid and efficient endocytosis of multiple raft components including ganglioside GM1, GPI-linked proteins and cholesterol, leading to dramatically decreased plasma membrane order^{2, 11}. Rafts are endocytosed through caveolae in a process that requires dynamin-2 and phosphorylation of a small fraction of caveolin-1 on tyrosine14 (pY14Cav1)³. The resultant loss of plasma membrane anchoring sites downregulates Rac1, Erk and PI-3-kinase-dependent pathways. Integrin-specific re-adhesion triggers trafficking of raft components back to the plasma membrane and restores anchorage-dependent signaling.

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The small GTPase Arf6 regulates endocytosis, post-endocytic recycling, exocytosis and cytoskeletal organization^{12, 13}. Importantly, Arf6 and several of its regulators are commonly over-expressed in metastatic cancers and strongly implicated in control of cell migration and spreading¹⁴. Consistent with this role, activation of Arf6 leads to increased Rac1 GTP-loading and movement of Rac1 to the plasma membrane^{15, 16}.

Microtubules (MT) also modulate Rac activity and function, cell spreading and migration^{17–19}. They do so in part by serving as tracks for intracellular vesicular trafficking. Additionally, MTs directly bind regulators of Rho family GTPases such as GEFH1²⁰.

In this study, we further examined the adhesion-dependent endocytosis and exocytosis of raft components in fibroblasts using a well-characterized lipid raft marker, cholera toxin subunit B (CTxB), which binds ganglioside mannoside 1 (GM1). Our results identify key components of this trafficking pathway and unexpectedly reveal the mechanisms by which microtubules and Arf6 regulate cell spreading.

RESULTS

Cytoskeletal regulation of raft endocytosis

We first tested the role of F-actin and microtubules in the caveolin-dependent endocytosis of rafts in mouse embryo fibroblasts (MEFs) after their detachment from the tissue culture plastic substratum where they adhere to a matrix composed mainly of fibronectin (FN). Cells were prelabeled with cholera toxin B (CTxB) while still adherent. Some cells were first pretreated with the actin depolymerizing drug latrunculin A (LatA) (1 μ M) or the microtubule depolymerizing agent nocodazole (NOC) (10 μ M), then detached and held in suspension for up to 90min in the continued presence of drugs. In control cells, CTxB began to internalize immediately after detachment and moved to a distinct perinuclear location by 90min (Figure 1a). When CTxB was instead added 90 min after detachment, binding was minimal, indicating clearance of GM1 from the cell surface (Figure 1b). LatA prevented the complete internalization of GM1 in suspension, indicated by the retention of CTxB surface labeling (Figure 1b). F-actin is therefore required for internalization of rafts through this pathway.

Nocodazole had no effect on detachment-induced internalization per se, as indicated by the loss of CTxB surface labeling in suspended cells (Figure 1b). However, in prelabeled cells, internalized CTxB remained in the cell periphery, failing to move to the cell center (Figure 1a). When prelabelled control cells were fixed and stained for γ -tubulin, perinuclear CTxB was localised around the microtubule organizing center (MTOC) (Figure 1c) (Pearson's Coefficient = 0.72 ± 0.042). Thus, internalized CTxB appears to be transported toward the minus end of the microtubules to the MTOC.

Identification of the intracellular compartment

We next sought to identify the intracellular compartment to which CTxB localises in suspended cells. The Golgi's proximity to the MTOC, together with known trafficking of CTxB to this compartment in adherent cells^{21, 22} made it an obvious candidate. In suspended cells, endocytosed CTxB and the cis-Golgi marker GM130 appeared to co-localise (Figure. 2a). However, careful examination at higher resolution showed CTxB to be present in a distinct structure that was complementary to the GM130 stain (Figure. 2a) (Pearson's coefficient = 0.20 ± 0.05 ; significantly less ($p < 0.001$) than overlap seen with MTOC). This pattern could be seen most clearly when the intensity of the GM130 signal was gradually increased in merged images (lower panel of Figure 2a).

To confirm this result, cells were treated with brefeldin A (BFA), which induces vesiculation and dispersion of the Golgi. Although BFA clearly dispersed the cis-Golgi marker, GM130,

(Figure 2b) and the trans-golgi marker, TGN38, (data not shown), endocytosed CTxB was unaffected (Figure 2b). Furthermore, BFA did not noticeably inhibit cell spreading or return of CTxB to the cell surface in cells replated on fibronectin (FN) (supplementary Figure 1a). Finally, a kinase-dead mutant of protein kinase D (PKD-KD) (K618N) that blocks exit of newly synthesized proteins from the Golgi ²³ also had no effect on the movement of CTxB from the perinuclear pool (Figure 2c) to the cell surface in replated cells (supplementary figure 1c). It did, however, block transport of the temperature sensitive VSV-GFP protein ²⁴ from the Golgi (supplementary figure 1d), confirming its efficacy. A PKD-KD (P155/287G) mutant not localized to the golgi was used as a control (supplementary figure 1b, 1d). VSV-GFP localized in the Golgi also showed minimal overlap with endocytosed CTxB. (supplementary figure 1e). Taken together, these data exclude the Golgi as the location of endocytosed GM1 in suspended cells.

SV40 internalizes through caveolae to the smooth ER (SER) ²⁵, thus, we examined this organelle. The SER marker, autocrine motility factor receptor (AMF-R) showed no colocalisation with endocytosed CTxB (Figure 2d) suggesting it is not trafficked to the SER.

Recycling endosomes (RE) localize near the MTOC and, in adherent cells, contain endocytosed rafts ^{22, 26}. To test this location, we utilized the well-characterised RE marker Rab11 ^{27, 28}. Endocytosed CTxB in suspended cells showed strong overlap with GFP-Rab11 (Figure 3a) (Pearsons coefficient 0.72 ± 0.06 , significantly higher ($p < 0.001$) than overlap seen with GM130). Moreover, Rab11 localisation and its overlap with CTxB were resistant to BFA (data not shown). We also examined internalized transferrin (Tf), which is efficiently trafficked to the RE following clathrin-dependent uptake ²⁹. Adherent cells surface labeled with transferrin-Alexa 594 (Tf-Alexa 594) were subsequently labeled with CTxB-Alexa 488 and detached. Endocytosed CTxB strongly co-localised with Tf-Alexa 594 at 30 min (Figure 3b lower panel) (Pearson's coefficient 0.75 ± 0.045 , significantly higher ($p < 0.001$) than overlap with GM130). The RE is therefore the site of internalized GM1 in suspended cells.

Rab11 regulates trafficking of TfR from recycling endosomes to the plasma membrane ²⁷. To test whether it similarly regulates GM1, we examined the effects of dominant negative S25N Rab11. Although S25N Rab11 blocked the exit of Tf from RE (Figure 3c, supplementary figure 2a), it affected neither the return of CTxB to the cell surface after replating (supplementary figure 2b) nor cell spreading (Figure 3c). In cells pre-labeled with both CTxB-Alexa 488 and Tf-Alexa 594, these fluorophores showed little co-localisation during cell spreading (supplementary Figure 1f). Thus, although both GM1 and Tf traffic from the RE to the plasma membrane during re-adhesion, they do so via distinct pathways.

Rab22a, also localizes to recycling endosomes and regulates trafficking of membranes endocytosed by clathrin-independent pathways ³⁰. However, dominant negative S19N Rab22 did not affect endocytosis of GM1 after detachment, nor its exocytosis or spreading of replated cells (supplementary data Fig. 2c, 2d). Thus, Rab22a does not appear to regulate this pathway.

Role of ARF6 in raft trafficking

Arf6 can regulate vesicular trafficking from the RE ³¹. Intriguingly, Arf6 was also reported to control movement of Rac1 from endosomes to the plasma membrane and Rac activation ^{15, 16}. This similarity to integrin-mediated adhesion prompted us to test the role of Arf6 in raft trafficking. In suspended MEFs, GFP-WT Arf6 strongly overlapped with endocytosed CTxB-Alexa 594 in REs (Figure 3d) (Pearsons Coefficient $= 0.71 \pm 0.004$, significantly higher ($p < 0.001$) than overlap with GM130). Surface CTxB in spreading cells also co-localised extensively with Arf6 at lamellipodia and membrane ruffles (Figure 3e).

We next tested the effects of dominant negative T27N Arf6, which blocks both cell spreading^{15, 32} and trafficking of some components out of the RE³¹. To determine whether effects on spreading were mediated by trafficking of lipid raft components, we examined MEFs from both caveolin1^{-/-} (Cav1^{-/-}) mice and matched WT littermates. Unlike WT, Cav1^{-/-} MEFs do not internalize raft markers after detachment and retain functional, membrane localised active Rac1 even in suspension². Thus, spreading of Cav1^{-/-} cells should not require return of raft components to the plasma membrane. Cells transiently transfected with WT or T27N Arf6 were detached, held in suspension for 90min and replated on fibronectin. T27N Arf6 inhibited the spreading of WT cells as expected but had absolutely no effect on Cav1^{-/-} cells (Figure 4a, b). T27 Arf6 also inhibited the appearance of GM1 on the cell surface in re-adherent WT but not Cav1^{-/-} cells (Figure 4b). At later times after replating (≥ 45 min), however, spreading and surface GM1 in T27N-expressing recovered (supplementary Figure 2e, 2f). Thus, inhibiting Arf6 slows rather than completely blocks GM1 exocytosis and cell spreading. Inhibition by T27N Arf6 was not the result of changes in integrin levels or function, as binding of cells to fibronectin was unaffected (supplementary figure 2g). WT Arf6 had no effect on spreading, surface GM1 levels or raft endocytosis compared to untransfected cells (data not shown).

Specific knockdown of Arf6 (supplementary figure 3d, 3e) also slowed cell spreading and the appearance of GM1 on the surface of WT but not Cav1^{-/-} MEFs (Figure 4c, d). Surface levels of GPI-linked proteins, which also localize to raft domains, were similarly regulated in WT MEFs (supplementary Figure 3f). However, neither T27N Arf6 (Figure 4b, lower panel) nor Arf6 knockdown (data not shown) had any effect on GM1 endocytosis after detachment. Thus dominant negative Arf6 and Arf6 depletion both indicate a requirement for Arf6 in raft exocytosis during replating.

The Arf6-insensitivity of Cav1^{-/-} cells supports the conclusion that membrane trafficking underlies the Arf6 requirement, however, Cav1 can inhibit signaling through its scaffolding domain or perhaps other mechanisms³³. We therefore examined compared WT Cav1 and phospho-deficient Y14FCav1. This mutant contains an intact scaffolding domain and forms caveolae but does not support GM1 internalization in detached cells³. Transfection of Cav1^{-/-} cells with WT Cav1 restored sensitivity to T27N Arf6 whereas Y14F Cav1 was ineffective (supplementary Figure 3a, 3b). Together, these data suggest that Arf6 controls the adhesion-regulated recycling of rafts from the RE to the plasma membrane and identifies a novel Arf6-dependent, Rab11- and Rab22-independent pathway for transport of lipid rafts out of recycling endosomes in spreading cells.

Rac1 activation and membrane targeting

We next determined if the Arf6-dependent transport of lipid rafts is necessary for membrane targeting and activation of Rac1 during cell spreading^{34, 35}. As before, we compared WT and Cav1^{-/-} cells. In WT MEFs, Rac1 membrane targeting and activation were reduced in suspension (Figure 4e and f). After 20 min on FN, both Rac1 membrane targeting and activity were restored in cells expressing WT Arf6, whereas cells expressing T27N Arf6 showed a 50% decrease in Rac1 membrane association and an 80% decrease in activation. After 45min, however, Rac1 membrane targeting and activity were comparable in WT and T27N Arf6 cells (Figure 4e and f). No difference was observed in Rac1 activation and membrane association between untransfected and WT Arf6-transfected cells (not shown). Consistent with these results, cells eventually spread and surface GM1 levels recover in T27N Arf6 expressors (supplementary Figure 2e, 2f). In contrast to WT MEFs, Rac1 membrane levels and activation remained high in detached Cav1^{-/-} cells, did not change upon re-adhesion, and were insensitive to T27N Arf6 (Figure 4e, Figure 4f). These results show that ARF6-dependent membrane raft trafficking is required for adhesion-dependent regulation of Rac1.

Role of ARF6 activation

To further test the role of Arf6 in adhesion-dependent raft trafficking, we measured effects of adhesion to fibronectin (FN) on Arf6 activation using a pull down assay³⁶. Arf6 activity decreased more than 60 % after detachment and recovered rapidly on re-adhesion (Figure 5a). Arf6 behaved in a qualitatively similar manner in Cav1^{-/-} (supplementary data Figure 4a) and CHO cells³⁷.

We next tested whether Arf6 activation is sufficient to drive GM1 from the RE to the plasma membrane. Cells were transfected with WT Arf6 or an activated, fast-cycling mutant of ARF6 (T157A) that spontaneously exchanges nucleotide and shows lower toxicity than GTPase-deficient Q67L Arf6³⁸. Neither T157A nor WT Arf6 significantly affected surface GM1 levels in adherent cells (Figure 4c). The initial rate of GM1 endocytosis after detachment was also unaffected (supplementary Figure 4e). However, at later times in suspension, endocytosed CTxB was dramatically redistributed toward the cortical region in T157A Arf6 expressing cells, compared to WT Arf6 and control cells (Figure 5b). These data suggest that active ARF6 drives the exit of CTxB from RE's.

Careful examination of CTxB- Alexa 594 images in cells expressing T157A Arf6- showed most of the cortical CTxB in vesicles under the plasma membrane rather than at the cell surface (Figure 5b). To confirm this, cells expressing WT or active Arf6 were surface labeled with CTxB and the bound protein quantified by Western blotting (Figure 5c). Little change in surface levels of GM1 was observed in adherent cells in any of these conditions. After 90min in suspension, surface GM1 decreased in all of the cells, with T157A ARF6 causing only a ~2 fold increase in surface GM1 compared to control suspended cells, which was still ~2.6 fold less than in adherent cells. HA-T157A ARF6 expressors were then pre-labeled with CTxB, replated, fixed and stained for HA. CTxB strongly co-localized with T157A ARF6 in distinct vesicles, plasma membrane ruffles and lamellipodia (Figure 5d) (Pearson's coefficient = 0.69 ± 0.04). We also noticed that T157A Arf6 expressors spread faster than control cells (supplementary data Figure 4b) and had marginally higher Rac1 activation, though Rac1 membrane association was similar (supplementary data Figure 4c 4d). Taken together, these results indicate that adhesion-dependent activation of Arf6 drives exit of rafts from the RE during cell spreading. However, additional adhesion-dependent steps may be needed for their efficient fusion with the plasma membrane.

Microtubules facilitate raft return to the plasma membrane

Since MTs mediate the movement of CTxB to the RE and also target active lamellipodia³⁹, we asked whether MTs might regulate movement of raft components back to the plasma membrane during spreading. Cells were pre-labeled with CTxB, replated on FN and stained for β -tubulin. CTxB-containing vesicles lying between the RE and the cell edge almost always co-localised with microtubules (Figure 6a). To functionally test this association, cells were treated with nocodazole (NOC). WT and Cav1^{-/-} MEFs were again compared to assess the importance of lipid raft trafficking. We also took advantage of the fact that when cells were pretreated with NOC while adherent (pre) and then detached, CTxB remained in the cortical region (Figure 1a) with little localization to Rab11 positive RE (supplementary Figure 5a) (Pearsons coefficient = 0.15 ± 0.05). In contrast, addition of NOC 90 min after detachment (post) showed endocytosed CTxB in dispersed Rab11-positive structures (supplementary Figure 5b) (Pearsons coefficient = 0.50 ± 0.05). GM1 after both treatments was, however, removed from the surface (supplementary Figure 5b). When replated on FN, spreading of WT cells was strongly inhibited by pre NOC but only moderately by post NOC (Figure 6b). By contrast, Cav1^{-/-} cells were barely affected by either treatment. Post NOC treatment of WT MEFs also strongly decreased surface GM1 levels as compared to pre NOC treated or control cells (Figure 6c). Post NOC also lowered Rac1 membrane targeting and activation, compared to untreated

or pre NOC cells (supplementary Figure 5c, supplementary Figure 5d). Cav1^{-/-} cells were again resistant (supplementary Figure 5c, supplementary Figure 5d). The morphology of the NOC-treated cells was not entirely normal but this result is consistent with MTs affecting the cytoskeleton through additional mechanisms such as focal adhesion turnover and regulation of Rho GTPases^{40, 41}. Nevertheless, these results show that their role in exocytic trafficking of rafts in re-adherent cells is an important mechanism by which MTs regulate cell spreading.

DISCUSSION

Previous studies showed that GM1-enriched lipid rafts were removed from the plasma membrane through caveolar endocytosis when anchorage-dependent cells were detached from the substratum³. Endocytosed rafts eventually coalesced in a distinct perinuclear region³. Integrin-mediated adhesion triggered their return to the plasma membrane. We now define this compartment as the recycling endosome (RE) and show that in suspended cells, endocytosed rafts are transported there in a microtubule-dependent manner. Adhesion to fibronectin triggers rapid recycling of these membranes out of the RE by a novel ARF6-dependent, Rab11- and Rab22-independent pathway, also along microtubules. Interestingly, we observed little or no co-localisation of rafts with caveolin in exocytic vesicles (Supplementary Figure 6a), indicating that this pathway is also caveolin-independent.

Arf6, however, appeared insufficient for efficient delivery of raft components back to the plasma membrane, with active Arf6 causing only a modest increase in their surface levels. Additional integrin-regulated step(s) may therefore be required for docking and/or fusion of exocytic vesicles with the plasma membrane. This sequence of events is diagrammed in figure 7.

In adherent cells under steady state conditions, endocytosis of GM1-enriched rafts can occur via clathrin-dependent, caveolin-dependent or clathrin- and caveolin-independent pathways^{26, 42–44}. In adherent cells, raft markers internalized through caveolae traffic prominently to the Golgi^{22, 45} SER²⁵ and partially to the RE²². By contrast, cell detachment triggers rapid and virtually complete internalization of raft components that are transported almost exclusively to the RE. This difference in postendocytic routing probably reflects the rapid uptake of a large volume of raft components through a specific pathway activated by detachment. Similarly, although multiple pathways may deliver rafts from RE's to the plasma membrane at steady state, this study identifies an Arf6-dependent, Rab11- and Rab22-independent pathway as the primary exocytic pathway triggered by re-adhesion. Indeed, the restoration of surface GM1, Rac1 function and spreading at later times after plating when Arf6 is inhibited are consistent with the existence of additional, less efficient exocytic pathways.

Previous studies identified lipid rafts as membrane binding sites for active Rac^{2, 46} and probably related small GTPases⁴. Rafts are enriched in lamellipodia and are required for cell spreading^{1, 2, 5}. Our data show that blocking return of rafts to the plasma membrane by inhibiting Arf6 function or MT assembly inhibited Rac1 membrane targeting and activation, and concomitantly impaired cell spreading in WT MEFs. Importantly, Cav1^{-/-} cells or Y14F Cav1-expressing cells, where rafts are not internalized after detachment, are resistant to these inhibitors. Thus, transport of rafts to the leading edge emerges as a crucial mechanism by which both Arf6 and MTs control Rac-mediated cell spreading.

Microtubule inhibitors have highly variable effects on cell spreading, depending on the cell type and exact protocol used^{17, 18, 47–50}. Our studies show that Cav1 expression, its phosphorylation (Y14) and the timing of drug treatment could account for differences in effects of MT disruption on lipid raft trafficking and hence cell spreading. Interestingly, both disruption of MTs and loss of Cav1 induce defects in polarity during migration (Grande et. al.

and ^{14, 39, 54}. It is therefore tempting to speculate that MT-dependent transport of rafts to lamellipodia contributes to polarity during migration. Indeed, Rac1 membrane binding sites within rafts localize to the lamellipodial edges ^{2,6,10,5}.

Arf6 was reported to regulate cell spreading by controlling the trafficking of Rac1 from a perinuclear compartment to the plasma membrane ^{15, 16}. The nature and significance of this minor perinuclear pool of Rac1 was unclear, especially since abundant Rac1 is present in the cytoplasm bound to RhoGDI and can exchange rapidly with membrane-bound Rac1 ⁵¹. Our data suggest that it is not the delivery of Rac1 per se that Arf6 regulates, but rather the delivery of Rac1 membrane binding sites in lipid rafts. These data do not exclude additional modulation of Rac1 activity by Arf6 through regulation of Rac GEFs ⁵², GAPs or direct binding of Arf to Rac ⁵³. Arf6 could also affect Rac GEFs through effects on membrane targeting sites in lipid rafts (Prag 2007 Mol Biol Cell. 2007 18:2935–2948?). Thus, trafficking of rafts appears to be a major regulatory pathway by which Arf6 controls Rac1 activation and cell spreading. These results differ from previous studies where growth factor activation of Rac did not require cell adhesion (del Pozo 2000), suggesting that different GEFs or GAPs have distinct requirement for raft membrane binding sites.

This study raises many new questions. Which motors drive centripetal movement of vesicles during endocytosis and centrifugal movement during exocytosis? How do raft and non-raft components co-exist in the RE? How do membrane binding sites in lipid rafts regulate specific Rac GEFs and GAPs? Which Arf6 GEFs and effectors trigger formation of raft-enriched exocytic vesicles from the RE during replating? How are they regulated by integrins and which additional integrin-regulated pathways control the final step of fusion with the plasma membrane? Their answers are likely to illuminate a variety of integrin-regulated processes.

MATERIALS AND METHODS

Reagents

Cholera toxin subunit B (CTxB) labelled with Alexa 594 (C22843) or Alexa 488 (C22841) were from Molecular Probes. Unlabelled cholera toxin B subunit (#227039), anti-cholera toxin antibody (#227040) and Brefeldin A (#203729) were obtained from Calbiochem. Latrunculin A (L5163), Nocodazole (M1404) and Alexa488-transferrin were from Sigma. Monoclonal anti-beta tubulin antibody (E7) was from Developmental Studies Hybridoma Bank. Anti-gamma tubulin and anti-caveolin1 antibodies were from Santa Cruz Biotechnology. Anti-GM130 conjugated to FITC and anti-phospho-caveolin (Tyr14) were purchased from Transduction Labs. Purified biotinylated aerolysin and anti-aerolysin antibody was from Protox Biotech. Anti Rac1 antibody was from (Upstate Biotechnology, Lake Placid, NY). Anti β 1 integrin antibody was obtained from Dr. A. F. Horwitz. Anti-AMF (autocrine motility factor) receptor antibody was obtained from Dr. Ivan Nabi. Anti Arf1, Arf3, Arf4 and Arf5 specific antibodies were obtained from Dr. Richard Kahn. Temperature-sensitive Vesicular Stomatitis Virus-G-GFP was obtained from Dr. Jenniffer Lipincott-schwartz. Protein kinase D1 kinase-dead mutant (PKD~KD), PKD~KD K618N and PKD~KD P155/287G mutant constructs were obtained from Dr. Vivek Malhotra. WTRab11-GFP, (S25N) Rab11, WT Rab22a, dominant negative (S19N) Rab22a, WT cav1, Y14F cav1, WT ARF6, dominant negative (T27N) Arf6, fast cycling (T157A) Arf6 constructs and the monoclonal anti-Arf6 antibody were as described ^{38, 55, 56, 30}. RNA interference sequence 5'-AGCTGCACCGCATTATCAA-3' of rat ARF6 mRNA (GenBank accession number NM_024152) used to generate complementary small interfering RNA (siRNA) oligonucleotides annealed and inserted into pSUPER.gfp/neo were as described ⁵⁷.

Tissue Culture

Mouse embryonic fibroblasts from Cav1^{-/-} and Cav1^{+/+} littermate mice (provided by Dr. Richard Anderson, University of Texas Health Sciences Center, Dallas TX) were cultured in DMEM medium with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen, Carlsbad, CA). Cells (10⁶) were transfected with 40µgm of DNA (5µgm DNA plasmid + 35µgm of salmon sperm DNA) by electroporation using the GenePulser Xcell (Bio-Rad Laboratories, Hercules, CA). Cells were incubated for 6h with 5mM sodium butyrate to promote protein expression. If required, cells were serum starved 12 h after electroporation in medium with 0.2% fetal bovine serum. Cells analyzed ~24 h after electroporation generally showed 90–95% transfection efficiency. 60µg of Arf6 or scrambled dsRNA in pSUPER vector were electroporated into cells, cells incubated for 72h and then serum starved as above.

For experiments, cells were detached with 1x trypsin, treated with soybean trypsin inhibitor, washed and held in suspension with 1% methylcellulose before replating on fibronectin-coated coverslips. For aerolysin labeling experiments cells were detached with Accutase (Innovative Cell Technologies Inc.) and processed as above. Fibronectin was used at 10µg/ml except for time course experiments where it was reduced to 2µg/ml to facilitate analysis of rates of exocytosis and spreading, as is indicated in figure legends.

Analysis of surface CTxB and aerolysin

Suspended or adherent cells were placed on ice for 15min and then incubated with 10µg/ml unlabelled CTxB (Calbiochem) or 1µg/ml of biotinylated aerolysin (Protox Biotech) for 30min. Cells were washed with cold PBS and 2×10⁵ cells were lysed in 100µl of SDS sample buffer. Cell-equivalent amounts of lysate were resolved by SDS PAGE, transferred to nitrocellulose and blocked with 5% nonfat dry milk in TBS+0.5% Tween-20 (TBS-T). Blots were incubated with anti-CTxB antibody (1µ/ml) or anti-aerolysin antibody (0.5µl/ml) followed by anti-goat or anti-mouse antibody conjugated to horse radish peroxidase respectively and developed using the ECL plus detection system (Amersham). Blots were stripped using Re-blot Plus stripping solution (Chemicon International) and re-probed with monoclonal anti-beta tubulin antibody as a loading control. Bands were quantified by densitometry using the Image J software. CTxB band intensities were normalized to tubulin.

Localisation of CTxB

Stably adherent cells were placed on ice for 15min then incubated with 10µg/ml of CTxB-Alexa 594 or 488 as indicated in Phosphate Buffered Saline (PBS) for 15 min. Cells were detached, held in suspension, replated as above and movement of endocytosed CTxB studied. In suspended or replated cells, surface GM1 was detected by incubating cells on ice with 10µg/ml CTxB-Alexa594 in PBS for 15min before fixation in 3.5% paraformaldehyde. Labeled cells were permeabilised in PBS containing 3% BSA and 0.05% Triton X100 for 15 min and blocked with 3%BSA in PBS for 1 h. Cells were then stained with anti AMF-R antibody, followed by anti-rat Alexa 488 antibody (Molecular Probes), with the anti gamma-tubulin antibody, followed by anti-mouse Alexa 488 antibody, or directly with FITC-conjugated anti-GM130 antibody. Cells expressing HA tagged Arf6 were incubated with anti-HA antibody, followed by anti-mouse IgG-Alexa488. For double labeling of CTxB-Alexa568 with tubulin, cells were fixed with cold 80% methanol for 10min at -20°C, stained with anti-β tubulin monoclonal antibody and anti-mouse IgG Alexa 488 secondary. Cells were mounted in Fluoromount-G (Southern Biotech), observed using the Zeiss LSM 510 laser confocal microscope with a 40x or 60x objective and analysed using either the Zeiss LSM Image Browser or the Image J software (NIH).

Quantitation of intracellular CTxB localisation

Cells were surface labeled with Alexa-CTxB, suspended, fixed and mounted as detailed above. Confocal images were recorded being careful to avoid pixel saturation. Using Image J software (NIH), thresholds were set to a) map the entire cell and b) to map the compact internal pool of endocytosed CTxB. The tracing tool was then used to select edges of the thresholded areas. Total intensity within the thresholded areas was determined, and the percentage of CTxB within the internal pool and the rest of the cell calculated.

Co-labelling of cells with transferrin and CTxB

Stably adherent cells were incubated with 20 µg/ml transferrin-Alexa594 (Sigma) for 30min, washed and chilled on ice for 15min. Cells were then incubated with 10 µg/ml of Alexa488-CTxB (Molecular Probes) in DMEM for 30min. Cells were washed with DMEM, detached and held in suspension with 1% methylcellulose in DMEM. At the end of the incubation they were fixed or replated on FN as described above.

Quantitation of endocytosed transferrin distribution

Cells labeled with transferrin-Alexa594 as above and replated were fixed, mounted and confocal images recorded being careful to avoid pixel saturation. Using Image J software (NIH), thresholds were set to a) map the entire cell and b) to map the endocytosed transferrin and the thresholded area for each calculated. Area occupied by transferrin was calculated as percentage of total cell area.

Drug treatments

Stably adherent cells were incubated for 30min with 1 µM Latrunculin A, detached with trypsin, held in suspension for 90 min with 1% methylcellulose, fixed or replated on FN and fixed with 3.5% paraformaldehyde. For BFA treatment, cells suspended for 90min were incubated for 30min with 1 µg/ml BFA in suspension and fixed immediately or replated on FN prior to fixation. For early NOC treatment, stably adherent cells were incubated for 10min with 10 µM NOC, detached with trypsin, washed with DMEM, and held in suspension for 90min with 1% methylcellulose in DMEM in the continuous presence of the drug. For late NOC treatment, 10 µM NOC was added to cells after 90 min in suspension. Control cells were left untreated. All cells were incubated for another 30min in suspension, washed to remove methylcellulose and replated on coverslips coated with 2 µg/ml FN with or without NOC. After 15 min, cells were washed, fixed and mounted as before. Cells were observed using a Nikon Diaphot TMD fluorescence microscope using a 60X oil objective and photographed using a Coolsnap HQ camera (Roper Scientific).

ARF6 activity assay

Arf6 activity was measured as previously described ³⁶. Stably adherent cells were detached, held in suspension 2h and replated on 10 µg/ml FN. Cells were lysed on ice in buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 1% Triton X-100, and protease and phosphatase inhibitor cocktails (Calbiochem). Lysates were centrifuged for 30min at 13,000xg and incubated with 40 µg GST-GGA3 plus 10 µl glutathione-Sepharose beads (Amersham) for 30 min. at 4°C. Beads were washed thrice in lysis buffer and eluted with SDS sample buffer. Arf6 was detected by Western blotting with anti Arf6 antibody ³⁶.

Subcellular Fractionation

Cells were washed with cold phosphate-buffered saline, scraped, and homogenized with 500 µl of buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 0.25 M sucrose, and a cocktail of protease inhibitors (Sigma, St. Louis, MO). Nuclei and unbroken cells were

removed by centrifugation at $1000 \times g$ for 10 min at 4°C , and the postnuclear supernatant was centrifuged at $100,000 \times g$ for 1 h at 4°C to sediment the membranes. 60% of membrane and 1.6% of cytosolic fractions were analyzed by Western blotting for Rac1 and $\beta 1$ integrin. Rac1 levels in the membrane fraction were normalized to $\beta 1$ integrin levels.

Rac1 activity assay

Rac1 pull down assays for GTP loading were performed essentially as described³⁵. 60% of GST-PBD bound active Rac1 and 1.6% of whole cell lysates were analyzed by Western blotting and bands quantified by densitometry. The amount of active Rac was calculated relative to total Rac for each sample.

Measuring cell spreading

Images of adherent cells were analyzed using Image J software. Thresholds were set to map the entire cell, the tracing tool was used to select the edge of each thresholded cell. The total area within the mapped edge for each cell was determined.

Quantitation of early CTxB endocytosis in suspended cells

Cells photographed using the Laser Confocal Microscope were analysed using Image J. By thresholding and using the “percentage shrink” macro, 20% of cell area from the cell periphery ($\approx 0.5\text{--}0.7\mu\text{m}$) was defined as cell edge. Central 40% area of the cell was defined as cell center. Intensities in these regions were measured, normalized to area and represented as percentage of total CTxB intensity.

Statistical significance

Comparison between data points were done using the Student's t test (Sigmaplot Statistical Analysis Software).

Fluorescent Image Colocalization Analysis

Images of cells photographed using the Laser Confocal Microscope at both wavelengths were analysed using Image J software (NIH), and Pearson's coefficient determined using the colocalization threshold plugin. Intensity quantitation along a defined line for each wavelength determined using the ‘plot profile’ function. List of values obtained were plotted in sigmaplot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The cytoskeleton in raft endocytosis

(a) Stably adherent MEFs pretreated with 1 μ M latrunculin A (LatA), 10 μ M nocodazole (NOC) or DMSO were surface labeled with CTxB-Alexa 488, then detached and held in suspension in the continued presence of drugs for the indicated times. (b) Unlabeled cells suspended for 90 min were surface labeled with CTxB-Alexa 594. Cells with no visible surface GM1 are marked by an asterisk. (c) Cells pre-labeled with CTxB-Alexa 488 (as in a) (GM1-CTxB) were suspended for 90 min then fixed and stained for γ -tubulin.

Figure 2. Endocytosed rafts do not localise to the Golgi or smooth ER

(a) Stably adherent MEFs surface labelled with CTxB-Alexa 594 (GM1-CTxB) were suspended for 90min then fixed and stained for the Golgi marker GM130 (GM130-FITC). Lower panel shows an enlarged view of the cell center with the intensity of GM130 (green) increased stepwise while the CTxB-Alexa 594 (red) is held constant. (b) Suspended MEFs with endocytosed CTxB-Alexa 594 (GM1-CTxB) were untreated (CONTROL) or treated with 10 μ g/ml brefeldin A (+BFA) for 30min. Cells were fixed and stained for GM130. (c) MEFs expressing GFP-tagged PKD-KD mutant (K618N) were surface labeled with CTxB-Alexa 594, detached for 90 min to allow endocytosis, then replated on FN (10 μ g/ml) for 30min. (d) MEFs

surface labelled with CTxB–Alexa 594 (CTxB) and incubated for 90 min in suspension were stained for autocrine motility factor receptor (AMF-R).

Figure 3. Endocytosed rafts target to recycling endosomes (RE)

(a) MEFs expressing Rab11-GFP were surface labeled with CTxB-Alexa 594 (CTxB) and incubated in suspension for 90 min. The central region is enlarged in the rightmost panel (b) Adherent MEFs labeled with transferrin-Alexa 594 (Transferrin) for 30 min were detached and surface labeled with CTxB-Alexa 488 on ice. Cells were fixed immediately (Susp 0') or warmed to 37°C for 30min (Susp 30'). The central region is enlarged in the rightmost panel (c) MEFs expressing Rab11 (S25N) -GFP were surface labelled with CTxB-Alexa 594 (GM1-CTxB), held in suspension for 90 min and replated on FN (10µg/ml) for 15min. Re-adherent cells were labeled with transferrin-Alexa 594 (transferrin) for 60min at 37°C (lower panels). Untransfected control cells marked by asterisks. (d) MEFs expressing GFP-WT Arf6 were

surface labeled with CTxB-Alexa 594 (GM1-CTxB) and incubated in suspension for 90 min. The central region is enlarged in the rightmost panel (e) Unlabeled GFP-WT Arf6 expressing cells suspended for 90 min were replated on FN (10 μ g/ml) for 15min and surface GM1 labeled with CTxB-Alexa594. Membrane ruffles are enlarged in the rightmost panel

Figure 4. Inhibition of Arf6 function

(a) Stably adherent WT and Cav1^{-/-} MEFs expressing HA-tagged WTArf6 (WT) or Arf6T27N (T27N) were suspended for 90min and replated on FN (2μg/ml) for 15min. Cells were then chilled, surface labeled with CTBx-Alexa 594, and images taken. Graph: surface areas were then measured; values are mean ± S.E in arbitrary units. (n=25 cells). Figure is representative of three independent experiments. (b) Cells suspended for 90min were replated on FN (2μg/ml) for 15 min. Suspended vs. replated cells were surface labeled with CTxB and bound CTxB detected by Western blotting (CTxB), using tubulin as a loading control (tubulin). Bands were quantified, values (means ± SE, n=3, normalized for tubulin) were calculated relative to WT Arf6-expressing WT MEFs. (c) Cells transfected with a vector coding for both GFP and Arf6

shRNA (KD) or scrambled control RNA (CON) were suspended, replated on FN (2 μ g/ml) for 15min, and labeled with CTxB-Alexa 594 (as in 4a). Graph: surface areas quantified; values are mean $\pm$ S.E in arbitrary units. (n=55 cells). Figure is representative of two independent experiments **(d)** Bound CTxB detected by Western blotting were quantified. Values (means $\pm$ SE, n=3, normalized for tubulin) were calculated relative to control WT MEFs. **(e)** WT and Cav1^{-/-} MEFs expressing HA-tagged WTArf6 (WT) or Arf6T27N (T27N) were suspended for 90min and replated on FN (2 μ g/ml) for 15 or 45min. Cells were lysed and fractionated as described in Methods. Rac1 in the membrane fraction was determined as described in Methods. Values are means $\pm$ SE in arbitrary units, n=3. **(f)** Active Rac1 was measured using the GST-PBD pull down assay and its percentage relative to total Rac1 calculated. Values are means $\pm$ SE, n=3.

Figure 5. Adhesion dependent activation of ARF6 promotes raft exocytosis

(a) WTMEFs that were stably adherent (SA), suspended for 90min (SUS), or replated on FN (10 μ g/ml) for 15 or 30min (FN15', FN30') were lysed and active ARF6 pulled down on GST-GGA3 beads (GGA-PD). GGA-PD and respective whole cell lysates (WCL) were Western blotted for Arf6 (WB: ARF6). Graph: bands were quantified and normalized intensities calculated relative to SA. Values are means $\pm$ SE, n=3. (b) MEFs expressing GFP WTARF6 (WT), HA-T157A Arf6 (T157A) or untransfected (control) were detached, surface labeled with CTxB-Alexa 594 (GM1-CTxB) and held in suspension for 90min. Images of cells with endocytosed GM1 were analyzed to determine the intensity in the central RE pool vs. the rest of the cell. Values are means $\pm$ SE, n=15, three independent experiments. (c) Suspended

untransfected (CON), WT and T157A Arf6-expressing cells were replated on FN (2 μ g/ml) for 15min. Cells were surface labeled with CTxB and bound CTxB detected by Western blotting (WB:CTxB). Tubulin served as loading control (WB:tubulin). CTxB band intensity was quantified, normalized to tubulin and calculated relative to adherent control cells. Values are means $\pm$ SE, n=3. **(d)** HA-T157A Arf6-expressing cells with endocytosed GM1-CTxB (as in 5b) were replated on FN (2 μ g/ml) for 15 min, fixed and stained for HA. Arrows indicate areas of co-labeling suggestive of transport vesicles and ruffling edges.

Figure 6. Microtubules in raft exocytosis

(a) WTMEFs pre-labelled with CTxB-Alexa 594 (GM1-CTxB) were held in suspension for 90 min and replated on FN (2μg/ml) for 15 min. Fixed cells were stained for β tubulin. Confocal images from multiple planes are shown as 3D reconstructions. Right panel shows boxed regions of interest (1, 2). (b) WTMEFs and Cav1^{-/-} MEFs were untreated (control), treated with nocodazole (10μM) before detachment (early NOC) or after being suspended for 90 min (late NOC). Cells were replated on FN (2μg/ml) for 15 min, fixed and stained for F-actin. Cell surface areas were determined from images. Values are means ± SE in arbitrary units; n=25 cells; three independent experiments gave similar results. (c) Re-adherent cells were incubated with CTxB and bound CTxB detected by Western blotting (WB:CTxB). Bands were quantified

and normalized for tubulin (WB:tubulin). Values are means $\pm$ SE relative to WT MEF control cells, n=3.

Figure 7. Summary

Cell detachment triggers actin-dependent endocytosis of rafts through caveolae. Vesicles are transported along microtubules to recycling endosomes (RE). Replating triggers activation of Arf6, which drives movement of rafts from RE along the microtubules to the cortical region of the cell. Other, unknown adhesion-dependent events complete the return of rafts to the PM.