A Unique Platform for H-Ras Signaling Involving Clathrin-independent Endocytosis

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Trafficking of H-Ras was examined to determine whether it can enter cells through clathrin-independent endocytosis (CIE). H-Ras colocalized with the CIE cargo protein, class I major histocompatibility complex, and it was sequestered in vacuoles that formed upon expression of an active mutant of Arf6, Q67L. Activation of Ras, either through epidermal growth factor stimulation or the expression of an active mutant of Ras, G12V, induced plasma membrane ruffling and macropinocytosis, a stimulated form of CIE. Live imaging of cells expressing H-RasG12V and fluorescent protein chimeras with pleckstrin homology domains that recognize specific phosphoinositides showed that incoming macropinosomes contained phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-trisphosphate (PIP₃). PIP₂ loss from the macropinosome was followed by the recruitment of Rab5, a downstream target of Ras, and then PIP₃ loss. Our studies support a model whereby Ras can signal on macropinosomes that pass through three distinct stages: PIP₂/PIP₃, PIP₃/Rab5, and Rab5. Vacuoles that form in cells expressing Arf6Q67L trap Ras signaling in the first stage, recruiting the active form of the Ras effectors extracellular signal-regulated kinase and protein kinase B (Akt) but not Rab5. Arf6 stimulation of macropinocytosis also involves passage through the distinct lipid phases, but recruitment of Akt is not observed.

INTRODUCTION

Ras GTPases regulate diverse cellular processes, including cell proliferation, differentiation, changes in gene expression, and apoptosis or survival (Boguski and McCormick, 1993). Activation of Ras links signaling from growth factor and G protein-coupled receptors with cytoplasmic effector pathways (Mor and Philips, 2006). There are three major Ras isoforms, H-Ras, N-Ras and K-Ras; yet, they all interact with a common set of effectors, including Raf, phosphatidylinositol 3-kinase (PI 3-kinase), and Ral guanine nucleotide dissociation stimulator. In light of the involvement of Ras in cellular processes and the role oncogenic Ras plays in tumorigenesis, it is important to understand the Ras signaling network and its regulation (Bos, 1995; Downward, 2003).

Ras proteins undergo posttranslational lipid modifications essential for their targeting and function (Hancock, 2003). Ras isoforms occupy different plasma membrane (PM) microdomains determined by carboxy-terminal farnesyl and palmitoyl modifications for H- and N-Ras and by the polybasic domain for K-Ras (Niv *et al.*, 2002; Prior *et al.*, 2003). In

addition to the PM, a distinct pool of H- and N-Ras resides on endoplasmic reticulum (ER) and Golgi membranes (Chiu *et al.*, 2002; Goodwin *et al.*, 2005; Rocks *et al.*, 2005). Phosphorylation of K-Ras causes it to translocate to mitochondria where it is involved in mediating apoptosis (Bivona *et al.*, 2006). Finally, Ras can generate signals from other compartments such as ramosomes (Rotblat *et al.*, 2006) and endosomes (Rizzo *et al.*, 2001; Jiang and Sorkin, 2002; Roy *et al.*, 2002).

There is good evidence that Ras can signal from endosomes (Hancock, 2003). Various studies have shown that H-Ras and activated epidermal growth factor (EGF) receptor are present on endosomes (Di Guglielmo *et al.*, 1994; Rizzo *et al.*, 2001; Jiang and Sorkin, 2002). Endosomes provide a unique environment for Ras signaling, because they are membrane compartments that undergo changes in lipid composition, cytoskeleton interaction, and binding of signaling and scaffold proteins. One study has implicated clathrin endocytosis in H-Ras signaling and demonstrated the H-Ras-induced activation of Raf1, but not PI 3-kinase, from these endosomes (Roy *et al.*, 2002). Other studies have demonstrated that activated H-Ras stimulates PM ruffling and macropinocytosis (Bar-Sagi and Feramisco, 1986) and that it leads to an increase in fluid endocytosis (Roberts *et al.*, 2000), but the nature of the endosomal membranes involved was not well defined.

Cells internalize PM by a variety of endocytic mechanisms that can be divided into two groups based on the dependence on clathrin coat protein. Clathrin-dependent endocytosis (CDE) is the pathway of entry for PM proteins that contain cytoplasmic sequences that bind to adaptor proteins to facilitate rapid endocytosis into cells (Conner and Schmid, 2003). Although there is evidence of distinctive types of endocytosis that do not require clathrin (Mayor and Pagano, 2007), we have been studying a clathrin-independent endocytic (CIE) pathway that is associated with the

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Abbreviations used: CD, cytochalasin D; CDE, clathrin-dependent endocytosis; CIE, clathrin-independent endocytosis; EGF, epidermal growth factor; MHCI, class I major histocompatibility complex; PH, pleckstrin homology; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PI 3-kinase, phosphatidylinositol 3-kinase; PIP 5-kinase, phosphatidylinositol 4-phosphate 5-kinase; PL, phospholipase; PM, plasma membrane; RBD, Ras binding domain; Tfn, transferrin; TFR, transferrin receptor.

Arf6 GTPase. Membrane proteins that lack cytoplasmic sequences for binding to clathrin adaptor proteins, including the class I major histocompatibility complex (MHCI) (Naslavsky *et al.*, 2003), the glycosylphosphatidyl inositol-anchored protein CD59 (Naslavsky *et al.*, 2004), integrins (Brown *et al.*, 2001), and E-cadherin (Paterson *et al.*, 2003), are internalized by this CIE pathway. Although we have primarily studied this CIE pathway in HeLa and COS-7 cells, we have also observed it in A431, MCF7 and Caco2 cells. After internalization, the Arf6-associated endosomes that contain these cargo proteins fuse with early endosomes and then, cargo can either be routed to late endosomes for degradation, or it can be recycled back to the plasma membrane (Naslavsky *et al.*, 2003). In resting HeLa cells, the recycling of MHCI and CD59 back to the plasma membrane occurs via characteristic tubular endosomes emanating from the juxtanuclear region that lack cargo internalized via clathrin-dependent mechanisms, e.g., transferrin receptor (TfR) (Naslavsky *et al.*, 2004; Weigert *et al.*, 2004).

Stimulated activation of Arf6, mediated by expression of EFA6, an Arf6 guanine nucleotide exchange factor (GEF), leads to the formation of PM protrusions (Radhakrishna *et al.*, 1996) and internalization by macropinocytosis (Brown *et al.*, 2001). Because Arf6 activates phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase) (Honda *et al.*, 1999) that generates phosphatidylinositol 4,5-bisphosphate (PIP₂), Arf6 inactivation is then required to halt PIP₂ production to allow membrane to recycle (Brown *et al.*, 2001). By contrast, expression of Arf6Q67L or PIP 5-kinase increases internalization of membranes and cargo that then accumulate in PIP₂-enriched vacuolar structures, resulting in a block in further trafficking (Brown *et al.*, 2001).

Interestingly, Chou and colleagues have shown that a downstream effector of Ras in the Raf signaling arm, extracellular signal-regulated kinase (Erk) and a scaffold protein, kinase suppressor of Ras, KSR1, are present on these CIE membranes and that they regulate recycling (Robertson *et al.*, 2006). Furthermore, activation of Arf6 by EFA6 (Robertson *et al.*, 2006) or by expression of Arf6Q67L (Tague *et al.*, 2004) leads to activation of Erk. The finding that the Ras effector Erk traffics with clathrin-independent cargo led us to examine whether H-Ras travels with and influences this CIE pathway.

MATERIALS AND METHODS

Cells, Plasmids, and Transfection

HeLa and COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin at 37°C with 5% CO₂.

Arf6, Arf6T27N, and Arf6Q67L were in pXS or pEGFP plasmid (Radhakrishna and Donaldson, 1997). Green fluorescent protein (GFP)-Rab5 and mutants were from R. Lodge (Laval, QC, Canada). H-Ras and H-RasG12V are in pDCR, pEGFP, or monomeric red fluorescent protein (mRFP) plasmid (Rotblat *et al.*, 2004). The RBD-GFP was a gift from Mark Philips (New York University, New York, NY) (Bivona *et al.*, 2005). Pleckstrin homology (PH)-phospholipase (PL) C δ was in pEGFP or pECFP and PH-protein kinase B (Akt) in mRFP were from Tamas Balla (National Institutes of Health, Bethesda, MD). Hemagglutinin (HA)-PLD2 was in pCGN and was from Mike Frohman (Stony Brook University, Stony Brook, NY). Akt1 in pEGFP-N3 was provided by Morris Birnbaum (University of Philadelphia, Philadelphia, PA). GFP-tH encoding GFP fused to the double palmitoylated and farnesylated carboxy terminal tail of H-Ras was from Clontech (Mountain View, CA), and tH was also appended onto the monomeric RFP vector from Roger Tsien (University of California-San Diego) forming RFP-tH. Flag-EFA6 was described previously (Brown *et al.*, 2001). For transfection, cells were plated and transfected the next day by using FuGENE (Roche Diagnostics, Indianapolis, IN). Experiments were performed 17–20 h after transfection.

Reagents and Antibodies

Rabbit polyclonal antibody to Arf6 (Song *et al.*, 1998) and mouse monoclonal antibody (mAb) to human MHCI (W6/32) (Naslavsky *et al.*, 2003) were described previously. The mouse monoclonal anti-HA antibody 16b12 was

purchased from Covance (Berkeley, CA). Monoclonal anti-early endosomal antigen (EEA1) was purchased from BD Biosciences (Palo Alto, CA). Monoclonal anti-phospho-Erk and polyclonal anti-phospho-Akt were purchased from Cell Signaling Technology (Danvers, MA). Invitrogen (Carlsbad, CA) was the source for transferrin (Tfn) conjugated to Alexa-633, rabbit polyclonal antibody to GFP, and all secondary antibodies conjugated to Alexa-594, -488, and -647. Cytochalasin D (CD) and EGF were purchased from Sigma-Aldrich (St. Louis, MO).

Immunofluorescence and Live Cell Imaging

For uptake of Tfn and MHCI, cells were serum starved for 30 min at 37°C in DMEM alone, fluorescently labeled Tfn and anti-MHCI (30–50 μ g/ml) were added, and cells were incubated for 30 min at 37°C. At the end of incubation, PM-associated ligand and antibodies were removed by rinsing the cells in low pH solution (0.5% acetic acid and 0.5 M NaCl, pH 3.0) for 20–30 s. Cells were then fixed with 2% formaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 min, and internalized MHCI antibody was labeled with 594-goat-anti-mouse immunoglobulin (Ig)G in the presence of 0.2% saponin. For indirect immunofluorescence, cells were fixed as described above and immunostained as described previously (Naslavsky *et al.*, 2003). Briefly, after fixation cells were incubated for 1 h with primary antibody diluted in 10% fetal calf serum in PBS in the presence of 0.2% saponin. After washing, cells were incubated for 1 h with secondary antibody diluted as described above.

For live cell imaging, HeLa or COS-7 cells were plated onto Lab-Tek coverglass chambers (Nalge Nunc International, Rochester, NY) and transfected with the indicated constructs. Eighteen hours after transfection, cells were imaged on a 37°C stage in CO₂-independent media. For single- or double-channel movies, images were acquired every 6 or 10 s, respectively. All images were obtained using a 510 LSM confocal microscope (Carl Zeiss, Thornwood, NY) with 63 $\times$ 1.3 numerical aperture PlanApo objective. After acquisition, images were handled using Adobe Photoshop (Adobe Systems, San Jose, CA). All experiments were confirmed at least three times, and a representative image is shown. Videos were generated using MetaMorph (Molecular Devices, Sunnyvale, CA).

Ras Binding Domain (RBD) Assay for GTP-bound Ras

To examine Ras-GTP levels, H-Ras- or H-Ras- and Arf6Q67L-expressing HeLa cells were serum starved for 1 h and then stimulated with 100 ng/ml EGF for 10 min as indicated. Cell were lysed and subjected to the glutathione transferase (GST)-RBD pull-down assay as described previously (Niv *et al.*, 2002). Bound proteins were eluted from the beads by boiling in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and resolved by SDS-PAGE, followed by transfer to nitrocellulose membrane. Western blot was carried out using rabbit anti-GFP to detect tagged H-Ras and rabbit anti-Arf6 to detect Arf6Q67L and the appropriate infrared secondary antibodies. The Western blot was visualized and quantified using an Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE). Data from three independent experiments are shown as the average percentage of Ras pelleted on GST-RBD beads with error represented as $\pm$ SD.

RESULTS

H-Ras Associates with the CIE Pathway

To determine whether H-Ras was present on endosomal membranes, we followed the endocytosis of two endogenous PM proteins, MHCI and TfR, that are internalized by CIE and CDE mechanisms, respectively (Weigert *et al.*, 2004). We used an antibody to MHCI and fluorescently labeled Tfn to follow MHCI and TfR endocytosis in GFP-H-Ras-expressing HeLa cells. After 30 min of internalization, H-Ras colocalized with MHCI, but not TfR, on peripheral endosomal structures and on distinctive, tubular recycling endosomes (Figure 1A), which we have shown carry MHCI and other CIE cargo back to the PM (Naslavsky *et al.*, 2003; Weigert *et al.*, 2004). MHCI was also observed on converged, juxtanuclear endosomes that contained Tfn as observed previously (Weigert *et al.*, 2004). The colocalization of H-Ras with MHCI, and not with Tfn, on both incoming and recycling tubular endosomes demonstrates that Ras is uniquely localized to this CIE pathway.

This CIE pathway is associated with the Arf6 GTPase, and we have shown that expression of Arf6Q67L causes a block in trafficking of clathrin-independent cargo immediately after internalization, resulting in cargo accumulation in vacuolar structures that are enriched in PIP₂ and actin (Brown *et al.*

Figure 1. H-Ras traffics with the CIE pathway. (A) Internalization of MHCI antibody and 633-labeled TfR for 30 min was performed in HeLa cells expressing GFP-H-Ras. Internalized MHCI was visualized with 594-labeled secondary antibodies. H-Ras was both associated with incoming MHCI-positive endosomes (arrowhead) and the characteristic CI recycling tubular endosomes that lack TfR (see inset). (B) Coexpression of RFP-H-Ras and Arf6Q67L-GFP was evaluated in live HeLa cells. Inset shows RFP-H-Ras localization to vacuoles. (C) HA-PLD2 was coexpressed with GFP-H-Ras in HeLa cells and processed for immunofluorescence as described in *Materials and Methods*. Inset shows HA-PLD2 and GFP-H-Ras colocalized at the PM and on the CI recycling tubular endosomes. (D) Activation of RFP-H-Ras in live COS-7 cells was observed using the Ras-GTP detector, RBD (Ras binding domain) fused to GFP. As soon as 30 s after EGF (100 ng/ml) application, the RBD was recruited to the PM. At 310 s, membrane ruffles were seen; at 390 s, a macropinosome was formed and H-Ras-GTP was on it as indicated by the RBD-GFP. Selected frames from a time-lapse movie (Supplemental Video 1) are shown. Note that in the figure, time between frames varies. Bars, 10 μ m. (E) H-Ras-GTP was assessed by a pull-down assay with GST-RBD. H-Ras or H-Ras- and Arf6Q67L-expressing cells were serum starved for 1 h, treated with EGF for 10 min as indicated, and subjected to the GST-RBD pull-down assay. The fraction of GTP-bound H-Ras is plotted as the average $\pm$ SD of three independent experiments.

et al., 2001; Naslavsky *et al.*, 2003). We examined the effect of expression of Arf6Q67L on Ras trafficking, and we found that H-Ras was at the PM and associated with the Arf6Q67L vacuoles (Figure 1B), similar to what is observed for CIE cargo (Naslavsky *et al.*, 2003). H-Ras association with Arf6Q67L vacuoles was also observed in MCF7, HT1080, and COS-7 cells (our unpublished data). It was recently shown that PLD2 plays a critical role in the activation of H-Ras via the EGF receptor and the T-cell receptor (Mor *et al.*, 2007; Zhao *et al.*, 2007). Intriguingly, in HeLa and COS-7 cells, the recycling of MHCI and other CIE cargo back to the PM is dependent upon the activation of Arf6 and its ability to activate PLD (Jovanovic *et al.*, 2006). We examined the distribution of transfected PLD2 in HeLa cells, and we found that it colocalized with wild-type H-Ras at the PM and on tubular endosomes (Figure 1C).

Next, we examined the effect of EGF treatment on Ras activation in live COS-7 cells cotransfected with a RBD-GFP chimera, which binds Ras-GTP specifically (Bivona *et*

al., 2005). Within 30 s of EGF addition, RBD-GFP was recruited from the cytosol onto the PM colocalizing with H-Ras in PM ruffles (Figure 1D and Supplemental Video 1). Shortly after this, these cells were actively ruffling, and, as a consequence, forming macropinosomes (Figure 1D and Supplemental Video 1) as has been reported previously (Bar-Sagi and Feramisco, 1986). H-Ras and RBD were both present at the PM and on the macropinosomes (Figure 1D and Supplemental Video 1), indicating that EGF stimulated Ras-GTP persisted on the macropinosome. In parallel experiments, we monitored biochemically the activation of H-Ras in HeLa cells upon addition of EGF by using the RBD pull-down assay. We found that very little of the overexpressed H-Ras was GTP-bound even when expressed alone, but EGF treatment led to a 10-fold increase in Ras-GTP (Figure 1E). Coexpression of H-Ras with Arf6Q67L did not lead to activation of H-Ras (Figure 1E). Similar results were also obtained with COS-7 cells (data not shown).

Figure 2. H-RasG12V traffics with the CIE pathway and stimulates macropinocytosis. (A) Internalization of MHCI antibody and 633-Tfn was performed in HeLa cells expressing GFP-H-RasG12V. Internalized MHCI was visualized with 594-labeled secondary antibodies. Macropinosomes were observed after 30 min of internalization and contained MHCI, but not Tfn (see inset). (B) GFP-H-RasG12V was expressed in HeLa cells, and selected frames from a time-lapse movie (Supplemental Video 2) are shown. Note that Video 2 is cropped. (C) HeLa cells expressing GFP-H-RasG12V were treated with 200 nM CD for 20 min, fixed, and examined. (D) GFP-H-RasG12V was expressed with Arf6T27N-HA in HeLa cells and processed for immunofluorescence. (E) RFP-H-RasG12V was coexpressed with Arf6Q67L-GFP in HeLa cells and imaged live. Bars, 10 μ m. (F) Quantification of proportion of tubular endosomes observed in cells expressing GFP-tH, GFP-H-Ras, GFP-H-RasG12V, and GFP-H-RasG12V after 20-min treatment with CD. Plotted are the average $\pm$ SD from two independent experiments.

H-RasG12V Induces Macropinocytosis, a Stimulated Form of CIE

Our observations that H-Ras was trafficking with the CIE pathway and that EGF treatment activated H-Ras and led to PM ruffling and macropinocytosis led us to examine whether we could also observe this with expression of the active Ras mutant (G12V). Expression of H-RasG12V induced membrane ruffling and macropinocytosis as observed in both fixed and live HeLa cells (Figure 2, A and B, and Supplemental Movie 2). This was also observed in COS-7, MCF7, and Chinese hamster ovary cells (unpublished data). We examined whether H-RasG12V-induced macropinosomes contained CIE cargo such as MHCI, and we found that the macropinosomes contained MHCI but not Tfn (Figure 2A). It was notable that MHCI and H-RasG12V colocalized extensively in the periphery and on incoming macropinosomes (Figure 2A, inset), whereas MHCI also colocalized with Tfn in more centrally located endosomes. Live cell imaging of cells expressing GFP-RasG12V revealed that these incoming macropinosomes were dynamic and turned over with time (Supplemental Video 2). The formation and turnover of macropinosomes and the absence of tubular endosomes is similar to what we had observed previously in cells expressing EFA6A (Brown *et al.*, 2001). These enlarged structures represent macropinosomes as they contained fluid phase markers and an increase in fluid uptake was observed in cells expressing RasG12V compared with cells expressing H-Ras (data not shown).

In resting cells, recycling of CIE membranes back to the PM is dependent on Arf6 activation of PLD (Jovanovic *et al.*, 2006) and actin polymerization (Weigert *et al.*, 2004). Macropinocytosis, in this case, is a form of stimulated CIE. This can be demonstrated by treatment of H-RasG12V-expressing cells with CD to inhibit actin polymerization (Figure 2C) or coexpression of dominant-negative Arf6T27N (Figure 2D), which resulted in the cessation of ruffling and macropi-

nocytosis, and the accumulation of H-RasG12V on tubular, recycling endosomes. Quantification of cells with tubular endosomes revealed that $\sim$ 30% of untransfected cells display MHCI tubular endosomes (data not shown); the same proportion of cells with tubular endosomes can be observed in cells expressing GFP-tH, which labels the tubular endosome, and in cells expressing GFP-H-Ras (Figure 2F). Few cells expressing H-RasG12V exhibit tubes; however, CD treatment resulted in the reappearance of Ras on tubular endosomes (Figure 2F). Interestingly, cells coexpressing Arf6Q67L continued to ruffle and form macropinosomes, and H-RasG12V was present both at the PM and on the accumulated vacuoles (Figure 2E). These results suggest that H-RasG12V, like the expression of EFA6 (Brown *et al.*, 2001), changes the membrane architecture of the CIE pathway and shifts the cargo flow from a constitutive to a stimulated form. At the same time, Arf6 activities can influence the cellular distribution of H-RasG12V.

H-RasG12V-induced Macropinosomes Undergo Changes in Phosphoinositide Composition

We previously showed that PIP₂ is lost from incoming EFA6-induced macropinosomes and that this loss is necessary for recycling of macropinosomal membrane back to the PM (Brown *et al.*, 2001). Because H-RasG12V induces macropinocytosis (Bar-Sagi and Feramisco, 1986) (this study) and activates PI 3-kinase to generate phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (Shields *et al.*, 2000), we examined the distribution of PIP₂ and PIP₃ in living cells. To do this, we expressed chimeric proteins of the PH domain of PLC δ fused to the green fluorescent protein (PH-PLC δ -GFP) for PIP₂ and the PH domain of Akt fused to RFP (PH-Akt-RFP) for PIP₃ in cells expressing H-RasG12V. The PH domain of Akt can also recognize phosphatidylinositol (3,4)-bisphosphate [PI(3,4)P₂], but here, for simplicity, we will refer to it binding to PIP₃. We found that the two PH domains colo-

Figure 3. Phosphoinositide dynamics on incoming macropinosomes. Untagged H-RasG12V was expressed in COS-7 cells to induce macropinocytosis together with PH-PLC δ -GFP and PH-Akt-RFP to observe the lipid content of macropinosomes. Selected frames from a time-lapse movie at 20-s intervals (Supplemental Video 3) are shown. Newly formed macropinosomes were labeled both with the PH-PLC δ -GFP and PH-Akt-RFP, indicating the presence of PIP₂ and PIP₃, respectively. Bars, 10 μ m.

calized at the PM, on membrane ruffles and on newly formed macropinosomes (Figure 3 and Supplemental Video 3). Although both PH domains were subsequently lost from the macropinosome, PH-PLC δ -GFP was always released before the release of PH-Akt-RFP (Figure 3 and Supplemental Video 3), suggesting that PIP₂ was lost from the macropinosome before PIP₃. In both cases the “loss” of PIP₂ and PIP₃ could be due to the conversion to another PIP form or destruction by hydrolysis. We quantified the frequency with which this sequential release of PH domains was observed, and we found that this change was observed in 75% of incoming macropinosomes that were observed forming within 2 μ m from the PM (60 of the 79 macropinosomes that began with both PH domains, lost PIP₂ followed by the loss of PIP₃; scored in five cells over a 5–10-min period). Although some macropinosomes did not change over the course of imaging, none of the observed macropinosomes lost PIP₃ before PIP₂ or simultaneously released both PH domains.

Rab5 Is Recruited onto Macropinosomes after the loss of PIP₂

Rab5 is a known effector of Ras proteins and both have been implicated in the regulation of endocytosis (Tall *et al.*, 2001).

Ras proteins can induce Rab5 activation through direct binding of Rin1, a Rab5 GEF (Tall *et al.*, 2001). To examine whether Rab5 was associated with the CIE pathway, we expressed wild type GFP-Rab5 with H-Ras or H-RasG12V in HeLa cells. When expressed with H-Ras, Rab5-positive endosomes were distributed throughout the cell with some localized to the juxtanuclear region (Figure 4A). The expression of H-Ras had no effect on Rab5 distribution, and the two GTP binding proteins did not colocalize. By contrast, in cells expressing H-RasG12V, Rab5 was recruited onto newly formed macropinosomes in the periphery (Figure 4A). However, we found that the Rab5 effector, EEA1 was not recruited onto peripheral macropinosomes suggesting that phosphatidylinositol-3-phosphate (PI3P) was absent from nascent macropinosomal membranes (Supplemental Figure 1). Given that the macropinosomes lacked Tfn and other clathrin-dependent cargo, these results suggest that H-RasG12V recruits Rab5 onto the CIE pathway. Having observed Rab5 recruitment onto macropinosomes in cells expressing H-RasG12V, we examined whether this could be observed in live H-Ras-expressing cells treated with EGF. Before EGF, GFP-Rab5 localized to perinuclear structures as seen in Figure 4A. Addition of EGF led to the recruitment of GFP-Rab5 onto newly formed macropinosomes in H-Ras-expressing cells (see Supplemental Figure 2 and Supplemental Video 10).

Rab5 recruitment on to membranes derived from CIE was intriguing to us, because Rab5 has been described as a component present on classical early endosomes that represent the convergence point for clathrin-dependent and clathrin-independent cargo (Naslavsky *et al.*, 2003, 2004). Furthermore, we do not observe Rab5 colocalizing with Arf6 or recruited to the Arf6Q67L vacuoles under any condition (Naslavsky *et al.*, 2003, 2004). Therefore, we suspected that Rab5 recruitment onto macropinosomes by H-RasG12V would be dependent on the lipid composition and that PIP₂ loss would be required before Rab5 recruitment. We coexpressed PH-PLC δ -CFP, GFP-Rab5, and an untagged H-RasG12V in COS-7 cells and, using live cell imaging, followed the dynamics of PIP₂ and Rab5 recruitment to newly formed macropinosomes. PH-PLC δ -CFP labeled the PM and incoming macropinosomes, and then it was subsequently released (Figure 4B and Supplemental Video 4), as we observed previously (Figure 3 and Supplemental Video 3). Between 10 and 20 s after PIP₂ release, Rab5 was recruited onto the macropinosome (Figure 4B and Supplemental Video 4). We found that PIP₂ loss was followed by Rab5 recruitment for 63% of the incoming macropinosomes (40 of 63 macropinosomes observed in 7 cells). Only newly formed (<2 μ m from PM) and PIP₂- but not Rab5-associated macropinosomes were counted. None of the observed macropinosomes demonstrated the opposite trend (i.e., Rab5 recruitment before PIP₂ loss or Rab5 and PIP₂ simultaneously present on incoming macropinosomes). These results demonstrate the Rab5 recruitment to the macropinosome by H-RasG12V is dependent on PIP₂ loss. We next examined whether PIP₃ was also released from macropinosomes before Rab5 recruitment by using GFP-Rab5 and PH-Akt-RFP. We found that Rab5 recruitment occurred before PH-Akt loss, suggesting that PIP₃ or PI(3,4)P₂, unlike PIP₂, was permissive for the association of Rab5 with macropinosomes (Figure 4C and Supplemental Video 5).

Macropinosome Maturation Provides Three Distinct Signaling Platforms for H-Ras

Our observations suggest that macropinosomes undergo distinct changes in protein and lipid composition after they enter cells. We can detect three separate stages of H-Ras-associated macropinosomes: PIP₂/PIP₃, PIP₃/Rab5, and

Figure 4. Rab5 recruitment onto macropinosomes by H-RasG12V is dependent on loss of PIP₂, but overlaps with PIP₃. (A) GFP-Rab5 was coexpressed in HeLa cells with either RFP-H-Ras (top row) or RFP-H-RasG12V (bottom row). RFP-H-Ras was localized to the PM and CI recycling tubular endosomes (see inset), whereas Rab5 was on discrete endosomes primarily in the juxtannuclear region. When coexpressed with RFP-H-RasG12V, Rab5 distribution was altered; it was recruited onto newly formed macropinosomes (see inset). (B and C) The recruitment of GFP-Rab5 onto macropinosomes formed in COS-7 cells expressing untagged H-RasG12V and either PH-PLCδ-CFP (PIP₂) (B) or PH-Akt-RFP (PIP₃) (C) was examined by live cell imaging (Supplemental Videos 4 and 5, respectively). Selected frames, at 20-s intervals, from a time-lapse movie are shown; arrowheads point to macropinosomes. CFP is pseudocolored red here. Bars, 10 μm.

Rab5 (Figure 5A). Because the incoming macropinosome contains PIP₂, and H-Ras traffics with this Arf6-associated, CIE pathway, we wondered whether expression of Arf6Q67L would capture this early signaling platform of H-Ras G12V (stage I). H-RasG12V localized to the PM and vacuoles in cells expressing Arf6Q67L (Figure 2E), and RBD was recruited onto both the PM and the vacuoles (Figure 5B), indicating that Arf6Q67L provides a suitable environment for active H-Ras to recruit its effectors.

Activation of Ras also leads to phosphorylation and activation of kinases including Erk and Akt. Antibodies detecting endogenous phospho-Erk and phospho-Akt also colocalized with H-RasG12V on these structures. Rab5, however, was not observed on these structures (Figure 5B) probably due to the

presence of PIP₂ (data not shown). By contrast, although wild-type H-Ras localized to the Arf6Q67L vacuoles, it was not activated there, because the RBD remained cytosolic (Figure 5C), and H-Ras-GTP was barely detectable by RBD pull-down assay (Figure 1E). Consistent with this, neither endogenous phospho-Erk nor phospho-Akt was observed recruited to H-Ras present on the vacuoles (Figure 5C). However, we detected an increase in phospho-Erk staining in cells expressing Arf6Q67L and wild-type H-Ras (compare phosphorylated [p] Erk staining in Figure 5C to pErk staining of untransfected cell marked with an arrow in Figure 5B) that was also seen in cells expressing Arf6Q67L alone (data not shown). This ability of Arf6-GTP to activate Erk has been observed by others (Tague *et al.*, 2004; Robertson *et al.*, 2006). However,

Figure 5. Model for macropinosome maturation and distinct signaling platforms for H-Ras. (A) The model depicts three distinct stages of H-RasG12V-induced macropinosome maturation, with specific lipid composition and unique signaling molecules they contain. Actin is required for the formation of a macropinosome (labeled in orange). PIP₂, PIP₃, and H-Ras* (indicating active H-Ras or H-RasG12V) are on the plasma membrane and on nascent macropinosomes. Expression of Arf6Q67L, which blocks CIE right after internalization, captures this newly formed macropinosome. In the second stage of maturation, the macropinosome loses the PIP₂ and acquires Rab5 that overlaps with PIP₃. At the last stage, PIP₃ is lost from the membranes, and Rab5 might contribute to the sorting of the cargo. H-Ras* was observed at all stages of macropinosomes maturation. (B and C) Arf6Q67L vacuoles capture the first stage in macropinosome maturation and help identify unique signaling molecules present on this platform. The distribution of different Ras effectors in cells expressing untagged Arf6Q67L and either H-RasG12V (B) or H-Ras (C). The RBD-GFP was recruited onto Arf6Q67L vacuoles by GFP-H-RasG12V, but not GFP-H-Ras (see insets, top rows, B and C). In B, arrow points to a nontransfected cell labeled with phospho-Erk antibody. Endogenous (active) pErk and pAkt were detected with antibodies on the vacuoles in the presence of GFP-H-RasG12V (insets, middle rows, B), but not GFP-H-Ras (insets, middle rows, C). Neither GFP-H-RasG12V nor GFP-H-Ras could recruit Rab5 onto the Arf6Q67L vacuoles (insets, bottom row, B and C). All images with individual effectors were taken with identical acquisition parameters. Bars, 10 μ m.

in the presence of H-RasG12V phospho-Erk staining was enhanced and localized to both the PM and the Arf6Q67L vacuoles, which suggests that H-RasG12V activates Erk on the vacuoles (Figure 5B). These results show that Arf6Q67L captures macropinosomes at a maturation stage where active H-Ras recruits certain effectors, such as Erk and Akt, but not others, such as Rab5 that require further maturation of the macropinosome.

Macropinosomes Generated through Activation of H-Ras or Arf6 Share Similarities in Phosphoinositide Composition and Differences in Effector Recruitment

The ability of Arf6Q67L to trap H-Ras-stimulated macropinosomes in the first stage of maturation (Figure 5A) led us to reexamine the phosphoinositide changes that occur when we activate Arf6 by expression of EFA6. We previously showed that expression of EFA6 stimulates ruffling and macropinocytosis and that loss of PIP₂ from the incoming macropinosome was required to recycle the membrane back to the PM (Brown et al., 2001). In cells expressing EFA6, both PH-PLC δ -GFP and PH-Akt-RFP were observed at the PM and on incoming macropinosomes and the PLC δ PH domain was lost from the macropinosome before loss of the Akt PH domain (Figure 6A and Supplemental Video 6), again suggesting that loss of PIP₂ preceded the loss of PIP₃ similar to what we observed with activated H-Ras. We quantified the frequency with which this sequential release of PH domains was observed, and we found that this change was observed in 73% of incoming macropinosomes that were observed forming within 2 μ m from the PM (13 of the 17 macropinosomes that began with both PH domains lost PIP₂, followed by the loss of PIP₃; scored in 9 cells). Although most macropinosomes showed this trend in lipid maturation over the course of imaging, some macropinosomes lost PIP₃ and maintained PIP₂. These macropinosomes were not dynamic and might reflect that under these conditions (EFA6 activa-

tion of Arf6), PIP 5-kinase may be continually activated. Indeed, under conditions of high EFA6 expression or coexpression of EFA6 plus Arf6, cells form vacuoles, mimicking the phenotype of cells expressing Arf6Q67L (unpublished observations) demonstrating once again that Arf6Q67L vacuoles are trapped macropinosomes.

Remarkably, in EFA6-expressing cells coexpressing PH-PLC δ -CFP and Rab5-GFP, we also saw that Rab5 was recruited onto the macropinosome subsequent to the loss of PIP₂ (Figure 6B and Supplemental Video 7). We found that PIP₂ loss was followed by Rab5 recruitment for 72% of the incoming macropinosomes (16 of 22 macropinosomes observed in 3 cells). Only newly formed (<2 μ m from PM) and PIP₂- but not Rab5-associated macropinosomes were counted. None of the observed macropinosomes demonstrated the opposite trend (i.e., Rab5 recruitment before PIP₂ loss or Rab5 and PIP₂ simultaneously present on incoming macropinosomes).

The similarity of the Ras-GTP and Arf6-GTP-induced macropinosome phosphoinositide composition was unexpected given that Ras activates PI 3-kinase and Arf6 activates PIP 5-kinase. This suggests that macropinocytosis induced through different mechanisms involves changes in both PIP₂ and PIP₃ during maturation and the subsequent recruitment of Rab5. In contrast, one would expect that H-Ras would recruit some specific effectors to macropinosomes that Arf6-GTP would not. Having used the PH domain from Akt, an effector of H-Ras, for detecting PIP₃, we next examined whether full length Akt could be observed on incoming macropinosomes generated by activation of H-Ras or Arf6. In cells expressing RFP-H-RasG12V, Akt1-GFP colocalized with Ras at the PM, and it was present for a time on the incoming macropinosome and then lost (Figure 7A and Supplemental Video 8) similar to the observed loss of the PIP₃ from the macropinosome. In cells expressing EFA6, in contrast, Akt1-GFP was present on the ruffling PM but not on the incoming macropinosome labeled

Figure 6. Macropinosomes generated by EFA6 contain PIP_2 and PIP_3 , and they acquire Rab5 after loss of PIP_2 . (A) EFA6 was expressed in COS-7 cells to induce macropinocytosis together with PH-PLC δ -GFP and PH-Akt-RFP to observe the lipid content of macropinosomes. Selected frames from a time-lapse movie at 20-s intervals (Supplemental Video 6) are shown. Newly formed macropinosomes were labeled both with the PH-PLC δ -GFP and PH-Akt-RFP, indicating the presence of PIP_2 and PIP_3 , respectively. (B) The recruitment of GFP-Rab5 onto macropinosomes formed in COS-7 cells expressing EFA6 in the background and PH-PLC δ -CFP (PIP_2) was examined by live cell imaging (Supplemental Video 7). Selected frames, at 20-s intervals, from a time-lapse movie are shown; arrowheads point to macropinosomes. CFP is pseudocolored red here. Bars, 10 μm .

with RFP-tH (Figure 7B and Supplemental Video 9). Together, this demonstrates that the stimulated macropinocytosis induced by activation of Arf6 or Ras involves similar stages of maturation in terms of phosphoinositide composition and Rab5 recruitment but differences in effector recruitment and activation.

DISCUSSION

To understand Ras signaling better, attention has focused on identifying unique cellular platforms for generating specific biological outputs (Hancock, 2003). Although H-Ras has been observed on endosomes (Rizzo *et al.*, 2001; Jiang and Sorkin, 2002; Roy *et al.*, 2002), the nature of those endosomal membranes was not thoroughly characterized. Here, we show that H-Ras associates with an endosomal pathway not involving clathrin that provides a unique platform for H-Ras signaling. These clathrin-independent endosomes through which H-Ras traffics contain cholesterol, phosphatidylinositides, and GPI-anchored proteins (Brown *et al.*, 2001; Naslavsky *et al.*, 2004); thus, they could provide discrete intracellular microenvironments for Ras signaling. The presence of other signaling molecules related to Ras, including Erk, the scaffold protein KSR (Robertson *et al.*, 2006), Src (Brown *et al.*, 2001), PLD2 (this study), and Arf6 (Radhakrishna and Donaldson, 1997) may facilitate H-Ras function not only through signaling but also by regulation of endocytosis and recycling in this CIE pathway. Arf6 regulates the movement of membrane into and out of this CIE pathway, alters the PM actin cytoskeleton, and has been implicated in cell migration and wound healing (D'Souza-Schorey and Chavrier, 2006). Indeed, alteration in levels of Arf6 and its regulators have been reported in many tumor metastasis models (Sabe,

2003). Ras is frequently mutated in cancer, resulting in an active protein (Shields *et al.*, 2000). Interestingly, other signaling molecules, such as Erk and Src, that traffic with the CIE have also been implicated in unregulated cell growth and tumorigenesis (Silva, 2004; Roberts and Der, 2007).

The macropinosomes that form during activation of H-Ras pass through three successive stages, providing an opportunity for three distinct platforms for Ras signaling. Initially, the H-RasG12V-stimulated macropinosomes have both PIP_2 and PIP_3 . Next, they lose PIP_2 , acquire Rab5, and then lose PIP_3 . We were also able to show that EGF stimulation of wild-type H-Ras led to its activation, the formation of macropinosomes (Figure 1), and the acquisition of Rab5 on the internalized macropinosome (Supplemental Figure 2). We show that H-RasG12V can recruit its downstream effectors phospho-Erk and phospho-Akt, but not Rab5, when trapped on Arf6Q67L vacuoles, which are arrested in the first stage (Figure 5). In the second stage, macropinosomes that lack PIP_2 but still have PIP_3 could recruit unique sets of Ras effectors, possibly reflecting the importance of the PI3-kinase arm of Ras signaling for tumor formation (Gupta *et al.*, 2007). Previous studies have shown that both activation of PI 3-kinase and PM ruffling are required for Ras-induced macropinosome formation (Li *et al.*, 1997; Barbieri *et al.*, 1998). Our study is in agreement with this, but it also shows that PIP_3 persists longer on the macropinosome than PIP_2 and overlaps with the recruitment of Rab5. We and others (Roberts *et al.*, 2000; Tall *et al.*, 2001) have observed that expression of H-RasG12V leads to the activation of Rab5, which can enhance endosome fusion. However, we demonstrate that the Rab5-associated macropinosomes in the periphery lack EEA1, a protein involved in early endosome fusion whose recruitment to membranes requires both Rab5 and a different phosphoinositide, PI3P. Indeed, this macropinosomal compartment

Figure 7. H-RasG12V- but not EFA6-expressing cells have Akt associated with the macropinosomes. (A) RFP-H-RasG12V was expressed in COS-7 cells to induce macropinocytosis together with Akt1-GFP. Selected frames from a time-lapse movie at 50-s intervals (Supplemental Video 8) are shown. Newly formed macropinosomes were labeled both with the RFP-H-RasG12V and Akt1-GFP after the loss of Akt1-GFP (arrowhead). (B) EFA6 was expressed in COS-7 cells to induce macropinocytosis together with RFP-tH that labels macropinosomes and Akt1-GFP. Selected frames from a time-lapse movie at 20-s intervals (Supplemental Video 9) are shown. RFP-tH and Akt1-GFP colocalized in membrane ruffles, whereas newly formed macropinosomes were not labeled with Akt1-GFP (arrowhead). Bars, 10 μ m.

may be related to the APPL compartment described by Zerial and colleagues that contains EGFR, Rab5, and distinct sets of Rab5 effectors but lacks EEA1 (Miaczynska *et al.*, 2004).

These findings reveal new relationships between the interactions between CIE and CDE and the role of Rab5 in these processes. Our model suggests that H-Ras and Rab5 separate at the final stage of macropinosome maturation (Figure 5A), with Ras and some MHCI recycling back to the PM and Rab5 and some MHCI moving in toward the juxtanuclear region. The fact that H-Ras never colocalizes with TfR coming in from CDE suggests that Ras leaves the Rab5 macropinosome before its eventual fusion with endosomes containing TfR. The trafficking of Ras out of the macropinosome suggests a fast lane of recycling of CIE cargo that bypasses convergence with the "classical" early endosome that warrants further investigation.

Our studies suggest that macropinocytosis, which can be induced by a variety of signals, represents a stimulated form of the constitutive CIE pathway. On stimulation, internalization changes from small pinosomes that enter independently of actin to large macropinosomes whose formation is actin-dependent. However, the types of cargo internalized

in both cases remains the same. Intriguingly, it is the activation of signaling molecules that we have shown reside in the CIE pathways (Arf6, Rac, Src, and Ras) (Radhakrishna *et al.*, 1999; Brown *et al.*, 2001; this study) that leads to macropinocytosis (Bar-Sagi and Feramisco, 1986; Nobes and Marsh, 2000; Brown *et al.*, 2001; Amyere *et al.*, 2002). Indeed, we were able to demonstrate that the macropinosomes formed upon EFA6A-induced activation of Arf6 also matured in terms of phosphoinositide content and Rab5 recruitment, but Ras-specific effectors were not present. Many others before us have recognized that macropinocytic vesicles contain cargo other than that internalized by CDE (Hewlett *et al.*, 1994; Veithen *et al.*, 1996). Furthermore, it is known that macropinocytosis is dependent upon PI 3-kinase (Araki *et al.*, 1996; Amyere *et al.*, 2000), phospholipase C (Amyere *et al.*, 2000), and PM cholesterol (Grimmer *et al.*, 2002). An interesting question is how the constitutive CIE pathway is altered upon switching to macropinocytosis.

Although the extent that cells engage in macropinocytosis and its physiological function is not yet known, these non-canonical endosomes could provide unique platforms for signaling, not only for Ras but also for Arf6, Rac, Src, and

others. Unlike the PM, the main site for signal transduction, the clathrin-independent endosomes or macropinosomes could provide a more isolated environment for the generation of unique signals. These internal, discrete structures can mature and change their lipid and protein composition providing spatiotemporal regulation of signal transduction.

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