Extracellular Signal-regulated Kinase Regulates Clathrin-independent Endosomal Trafficking[□]

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Extracellular signal-regulated kinase (Erk) is widely recognized for its central role in cell proliferation and motility. Although previous work has shown that Erk is localized at endosomal compartments, no role for Erk in regulating endosomal trafficking has been demonstrated. Here, we report that Erk signaling regulates trafficking through the clathrin-independent, ADP-ribosylation factor 6 (Arf6) GTPase-regulated endosomal pathway. Inactivation of Erk induced by a variety of methods leads to a dramatic expansion of the Arf6 endosomal recycling compartment, and intracellular accumulation of cargo, such as class I major histocompatibility complex, within the expanded endosome. Treatment of cells with the mitogen-activated protein kinase kinase (MEK) inhibitor U0126 reduces surface expression of MHCI without affecting its rate of endocytosis, suggesting that inactivation of Erk perturbs recycling. Furthermore, under conditions where Erk activity is inhibited, a large cohort of Erk, MEK, and the Erk scaffold kinase suppressor of Ras 1 accumulates at the Arf6 recycling compartment. The requirement for Erk was highly specific for this endocytic pathway, because its inhibition had no effect on trafficking of cargo of the classical clathrin-dependent pathway. These studies reveal a previously unappreciated link of Erk signaling to organelle dynamics and endosomal trafficking.

INTRODUCTION

Endocytosis and recycling play central roles in regulating the activity of cell surface proteins, such as growth factor receptors, cell adhesion molecules, and ion channels (Schmid, 1997). Endocytosis occurs through clathrin-dependent and -independent pathways, the latter of which includes phagocytosis, and caveolin- and lipid raft-mediated routes (Nichols and Lippincott-Schwartz, 2001; Johannes and Lamaze, 2002). The mechanism by which cargo is sorted is best understood for proteins internalized via the clathrin pathway (Kirchhausen, 1999, 2000). Sorting signals in the cytoplasmic domains of cargo are recognized by adaptor proteins, such as adaptor protein complex-2, which direct their recruitment into clathrin-coated pits (Bonifacino and Traub, 2003; Robinson, 2004; Sorkin, 2004). Endocytosed cargo are then targeted to early endosomes marked by Rab5 and EEA1, from which they may be sorted to lysosomes, to the biosynthetic pathway, or recycled to the plasma membrane. Cargo within this pathway traverse well-character-

ized compartments, and much of the machinery involved in regulating sorting has been identified.

By comparison, the mechanisms governing clathrin-independent endocytosis are relatively poorly understood (Nichols and Lippincott-Schwartz, 2001; Johannes and Lamaze, 2002). Recently, much attention has focused on the endosomal system regulated by the ADP-ribosylation factor 6 (Arf6) GTPase, which is clathrin-independent in many, if not most, cell types. This so-called Arf6 pathway mediates the internalization of proteins such as class I major histocompatibility complex molecules (MHCI), β 1 integrin, and E-cadherin and thus regulates processes as diverse as immune surveillance and cell adhesion (Donaldson, 2003). In addition, recycling through this pathway is essential for phagocytosis in macrophages (Zhang *et al.*, 1998; Niedergang *et al.*, 2003) as well as for cell spreading and lamellipodia formation (Song *et al.*, 1998; Radhakrishna *et al.*, 1999; Al-Awar *et al.*, 2000). Arf6 pathway cargo lack the sorting signals found in proteins endocytosed through the clathrin route. It is thought that cargo are internalized from regions of the plasma membrane enriched in cholesterol (Naslavsky *et al.*, 2004b). After endocytosis, cargo traverse a recycling compartment termed the tubular endosome (because of its prominent tubular morphology) that is distinct from the classical early and recycling endosomes mentioned above (Donaldson and Radhakrishna, 2001). Whereas recycling of cargo in the classical clathrin pathway is regulated by Rab4 (McCaffrey *et al.*, 2001; van der Sluijs *et al.*, 2001; Mohrmann *et al.*, 2002a,b) and Rab11 (Ullrich *et al.*, 1996; Ren *et al.*, 1998; Schlierf *et al.*, 2000), recycling from the tubular endosome to the plasma membrane is regulated by Arf6, Rab22, and Rab11 (Al-Awar *et al.*, 2000; Donaldson and Radhakrishna, 2001; Powelka *et al.*, 2004; Weigert *et al.*, 2004). Recent work

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Abbreviations used: Arf6, ADP-ribosylation factor 6; Erk, extracellular signal-regulated kinase; KSR, kinase suppressor of Ras; MHCI, class I major histocompatibility complex.

has shown that recycling through this latter pathway can be stimulated by extracellular stimuli (Powelka *et al.*, 2004), but the signaling pathways that regulate recycling have not been identified. Indeed, very little is known about regulation of recycling through clathrin-dependent or -independent endocytic routes.

Extracellular signal-regulated kinases 1 and 2 (Erk1/2) are central regulators of cellular proliferation, survival, and motility, and the mechanisms governing their activation have been extensively studied (Cheung and Slack, 2004; Roux and Blenis, 2004; Viala and Pouyssegur, 2004). Erk1/2 are activated by phosphorylation, mediated by the dual-specificity kinase MEK, which in turn is activated by the Raf kinase (Torii *et al.*, 2004b). Scaffold proteins play a key role in Erk activation by binding multiple components of the Raf-MEK-Erk module to promote signal transduction, amplification, and specificity.

The classical view of Erk regulation has held that Erk resides in its inactive state in the cytosol and is activated at the plasma membrane through recruitment of Raf by the Ras GTPase. Erk then translocates to the nucleus and phosphorylates multiple transcription factors to regulate gene expression, which underlies many of its biological effects. However, more recent work reveals that Erk is activated at additional subcellular organelles (Chiu *et al.*, 2002) and that scaffold proteins play a central role in this localized recruitment (van Drogen and Peter, 2002; Morrison and Davis, 2003; Yoshioka, 2004). For example, the Sef scaffold mediates activation of Erk at the Golgi complex (Philips, 2004; Torii *et al.*, 2004a). Fragmentation of the Golgi during mitosis is promoted by the Erk-MEK pathway and is thought to be mediated in part through phosphorylation of Golgi reassembly stacking protein 55 (Acharya *et al.*, 1998; Colanzi *et al.*, 2000; Jesch *et al.*, 2001). The MP-1 scaffold mediates activation of Erk at late endosomes (Teis *et al.*, 2002). However, in contrast to its role in regulating Golgi dynamics, Erk has not been shown to function in endosomal trafficking. The kinase suppressor of Ras (KSR) 1 scaffold promotes activation of Erk at the plasma membrane (Sundaram and Han, 1995; Therrien *et al.*, 1995; Michaud *et al.*, 1997; Stewart *et al.*, 1999). Contrary to its name, KSR1 is generally thought to be catalytically inactive as a kinase (Muller *et al.*, 2001; Roy *et al.*, 2002). Under quiescent conditions, KSR1 is complexed with inactive MEK in the cytosol. On mitogen stimulation, Erk binds to KSR1, and the Erk-MEK-KSR1 complex is recruited to the plasma membrane (Stewart *et al.*, 1999; Muller *et al.*, 2001; Ory *et al.*, 2003). Whether KSR1 promotes Erk activation at additional subcellular locations has not been examined.

We have been investigating the mechanisms that govern trafficking through the Arf6 pathway. In the current study, we demonstrate a novel role for Erk, MEK, and KSR1 in regulating dynamics of the tubular endosome and in the trafficking of Arf6 cargo. This work identifies a novel site of action for Erk, and reveals a specific role for this signaling module in clathrin-independent endosomal trafficking.

MATERIALS AND METHODS

Cell Culture

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, and GlutaMax (Invitrogen, Carlsbad, CA). Cultures were maintained at 37°C in 5% CO₂. Epidermal growth factor (EGF; Invitrogen) was used at a final concentration of 100 ng/ml. HeLa cells were transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) or LipofectAMINE 2000 (Invitrogen) as detailed below.

Plasmids and Constructs

FLAG epitope-tagged KSR1 and KSR1(C809Y) in pCMV5 have been described previously (Brennan *et al.*, 2002). The pleckstrin homology (PH) domain of phospholipase C δ in pEGFP was provided by Dr. Mark Lemmon (University of Pennsylvania School of Medicine, Philadelphia, PA) and has been described previously (Falasca *et al.*, 1998). Hemagglutinin (HA)-tagged Erk1/pCNA has been described previously (Chou and Blenis, 1996). FLAG-tagged ERK2 and ERK2(AEF) in pcDNA3 were kindly provided by Dr. Jiing-Dwan Lee (Scripps Research Institute, La Jolla, CA). HA-Arf6/pLNCX was provided by Dr. Morris Birnbaum (University of Pennsylvania School of Medicine). HA-TRE17(long)/pcDNA3 has been described previously (Martinu *et al.*, 2004).

Antibodies and Reagents

Antiserum against KSR1 phosphorylated on serine 392, anti-KSR(P392), has been described previously (Matheny *et al.*, 2004). Anti-MHCI (hybridoma W6/32) (Barnstable *et al.*, 1978) was used for immunofluorescence microscopy. Fluorescein isothiocyanate (FITC)-conjugated antibodies against human MHC1 (Biodesign International, Kennebunk, ME) and the transferrin receptor (anti-CD71; BD Biosciences Pharmingen, San Diego, CA) were used for fluorescence-activated cell sorting (FACS) analysis. Anti-Erk antibody for immunoblotting was provided by Dr. John Blenis (Harvard Medical School). Anti-Erk and anti-MEK antibodies for immunofluorescence microscopic analysis were purchased from BD Biosciences Transduction Laboratories (Lexington, KY). For immunofluorescence of HA-tagged proteins, anti-HA antibody from Santa Cruz Biotechnology (Santa Cruz, CA) (sc-805) or Roche Diagnostics (clone 12CA5) was used; for immunoblotting, the former was used. Anti-FLAG (clone M3 or M5) was purchased from Sigma-Aldrich (St. Louis, MO).

Secondary antibodies used were Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 633-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Alexa Fluor 546-conjugated transferrin (Molecular Probes) was used at 160 μ g/ml for 1 h. U0126 (Promega, Madison, WI) was dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 50 μ M.

Immunoblotting and GGA3 Pull-Downs

For immunoblotting experiments, HeLa cells were seeded at 4×10^5 cells/35-mm plate. The next day, cells were transfected with 4 μ g of total DNA and 7.5 μ l of LipofectAMINE 2000 per plate. Cells were subsequently treated as indicated and then lysed in 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.7 μ g of pepstatin per milliliter, and 1 μ g of leupeptin per milliliter. Samples were boiled, fractionated by SDS-PAGE, immunoblotted with the indicated antibodies, and then detected by enhanced chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

To monitor Arf6 activation in vivo, GGA3 pull-down assays were performed as described previously (Martinu *et al.*, 2004). Briefly, HeLa cells were cotransfected with HA-Arf6 and the indicated constructs. Cells were treated with U0126 or DMSO for 5 h before lysis and then subjected to pull-downs using recombinant glutathione S-transferase (GST)-GGA3 (15 μ g) for 1 h. The bound, active Arf6 was detected by anti-HA immunoblotting.

Confocal Immunofluorescence Microscopy

HeLa cells were seeded on 10-mm coverslips at a density of 2×10^5 cells/35-mm plate. The following day, cells were transfected using 2 μ g of total DNA and 6 μ l of FuGENE6 per plate. Twenty-four hours after transfection, cells were treated as indicated and then fixed as described previously (Martinu *et al.*, 2004). Coverslips were incubated with primary antibodies for 2–3 h at room temperature, washed, incubated with fluorescently labeled secondary antibodies for 1 h, and then washed twice in 10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100 and once in distilled water. Samples were mounted with SloFade (Molecular Probes) and viewed on a Zeiss confocal microscope with LSM510 software, using excitation wavelengths of 488 nm (FITC), 546 nm (Cy3), or 633 nm (Cy5).

For live cell imaging experiments, HeLa cells were seeded at 2×10^5 cells/35-mm glass-bottomed microwell dishes (MatTek, Ashland, MA) and transfected as described above. The next day, cells were visualized by confocal microscopy at 488 nm using LSM510 software. Cells were monitored for 1 h, collecting images every 30 s. Cells treated with U0126 were also monitored for 10 min before the addition of drug.

Flow Cytometry and Endocytosis Assays

HeLa cells were seeded at a density of 5×10^5 . The next day, cells were treated with U0126 or vehicle for 5 h and then resuspended using 10 mM EDTA/phosphate-buffered saline (PBS). Cells were pelleted and then resuspended in growth medium. Steady-state surface and total populations of MHC1 or transferrin receptor were measured as follows. To measure surface populations, cells were resuspended in growth medium (50 μ l) and incubated with FITC-conjugated anti-MHC I (0.5 μ g) or anti-transferrin receptor (0.25

Figure 1. The Erk scaffold KSR1 localizes to the Arf6 tubular endosomal compartment. (A) FLAG-KSR was transfected into HeLa cells and subjected to indirect immunofluorescence using anti-FLAG (left) or anti-P392 (middle) antibodies. (B) FLAG-KSR was co-transfected with GFP-PLC δ (PH); KSR was detected using anti-P392. (C and D) HeLa cells were transfected with FLAG-KSR1. Live cells were incubated in the continued presence of anti-MHCI for 4 h (C) or Alexa Fluor 546-conjugated-transferrin for 1 h (D). Cells were then fixed and probed with anti-P392. Bar, 10 μ m.

μ g) for 45 min at 4°C. As a negative control, FITC-conjugated antibody against murine MHCI (anti-H2D; 0.5 μ g) was used. Anti-H2D does not react with human cells and was used to determine nonspecific, background fluorescence. Cells were washed three times in FACS buffer (5% FBS and 0.02% sodium azide in PBS) and then fixed in PBS containing 2% formaldehyde for 15 min on ice. After fixation, cells were washed three times in FACS buffer, three times in Sheath fluid (Fisher Scientific, Pittsburgh, PA), and stored at 4°C until analysis. To measure total MHCI, resuspended cells were immediately fixed in PBS containing 1% formaldehyde for 15 min at room temperature. Cells were permeabilized by washing twice in medium containing 0.5% saponin and then incubated with FITC-conjugated anti-MHCI, anti-transferrin receptor, or anti-H2D for 45 min at 4°C as described above. Samples were washed three times in FACS buffer containing 0.5% saponin followed by three washes in Sheath fluid and then stored at 4°C until analysis. All data were processed using CellQuest Pro software (BD Biosciences, San Jose, CA). Mean fluorescence intensity signals obtained from anti-H2D staining were defined as background and subtracted from each experimental sample. The ratio of surface signal:total signal was calculated. This value for vehicle-treated control cells was defined as 1.0, and the values for U0126- and PD98059-treated samples were presented as a fraction of that in control cells. Data represent the results from between three and eight experiments, each performed in duplicate.

To measure initial endocytosis rates, cells were incubated with anti-MHCI antibody on ice for 45 min to label the surface population of MHCI. Cells were washed with ice-cold growth medium and then incubated for the indicated times at 37°C to allow internalization. At each time point, samples were

transferred to ice to halt trafficking, and FITC-conjugated anti-mouse antibody was added to detect the anti-MHCI remaining at the cell surface. Samples were then washed in ice-cold growth medium, fixed, washed, and analyzed as described above. All experiments were performed in duplicate.

RESULTS

The Erk Pathway Scaffolding Protein KSR1 Localizes to the Arf6-regulated Tubular Endosomal Compartment

KSR1 has been reported previously to translocate between the cytosol and plasma membrane (Muller *et al.*, 2001; Ory *et al.*, 2003; Matheny *et al.*, 2004). However, careful examination of published micrographs revealed that its cytosolic staining was not simply diffuse but partly on subtle punctate and tubular structures (Muller *et al.*, 2001; Matheny *et al.*, 2004). To further investigate this compartmentalization, we performed indirect immunofluorescence confocal microscopy. FLAG-epitope-tagged KSR1 was transfected into HeLa cells and then visualized with anti-FLAG antibody. Consistent with previous reports, KSR1 was detected at the plasma membrane and on tubular structures (Figure 1A). To deter-

Figure 2. KSR1 overexpression reduces plasma membrane and enhances tubular endosomal localization of Arf6 pathway markers. HA-tagged Arf6 or -TRE17(long) were expressed alone (A and B) or together with FLAG-KSR1 (C and D) in HeLa cells. Cells were subjected to indirect immunofluorescence using anti-HA and anti-P392 antibodies as indicated. Bar, 10 μ m.

mine whether recruitment to either compartment correlated with KSR1's activation state, samples were simultaneously probed with an antibody that specifically recognizes KSR1 that is phosphorylated on serine 392 (anti-P392). Previous work has shown that phosphorylation at this site inhibits recruitment to the plasma membrane, and it has therefore typically been used as a marker for the inactive state of KSR1 (Muller *et al.*, 2001; Brennan *et al.*, 2002; Ory *et al.*, 2003). Strikingly, compared with total KSR1, the anti-P392-reactive population was highly enriched on the tubular structures (Figure 1A). These observations indicate that phosphorylation of serine 392 of KSR1 correlates with its localization to the tubules.

The appearance of the tubular structures was highly reminiscent of the recycling tubular endosome controlled by the Arf6 GTPase. This compartment is enriched in phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] (Brown *et al.*, 2001), which can be visualized through its high-affinity interaction with the phospholipase C δ PH domain tagged with green fluorescent protein [GFP-PLC δ (PH)] (Fasasca *et al.*, 1998). To determine whether the tubular structures decorated by KSR1 correspond to the Arf6 tubular endosome, HeLa cells were cotransfected with FLAG-KSR1 and GFP-PLC δ (PH). As shown in Figure 1B, the anti-P392 staining precisely colocalized with GFP-PLC δ (PH). We also compared KSR1 localization with that of an endogenous cargo of the Arf6 pathway, MHCI (Brown *et al.*, 2001; Donaldson and Radhakrishna, 2001). HeLa cells were transfected with FLAG-KSR1, incubated in the continued presence of anti-MHCI antibody for 4 h, and then processed for indirect immunofluorescence microscopy. Figure 1C confirms that endogenous MHCI indeed colocalizes with phospho-392 KSR1. In contrast, no significant colocalization of KSR1 was observed with transferrin, a cargo of the classical clathrin-dependent endocytic pathway (Figure 1D). These data reveal a previously unrecognized localization of KSR1 specifically to the Arf6-regulated endosomal pathway.

KSR1 Overexpression Inhibits Plasma Membrane Localization of Markers of the Arf6 Pathway

In the experiments described above, two striking effects of KSR1 overexpression were noted. First, tubular elements were highly abundant in cells expressing KSR1, as monitored by using the GFP-PLC δ (PH) marker (Figure 1B). Approximately 70% of cells expressing KSR1 exhibited numerous tubules as typified in Figure 1B, and their abundance largely correlated with expression levels. Second, staining of

MHCI at the tubular endosome was greatly enhanced relative to the plasma membrane (compare KSR1-positive and -negative cells; Figure 1C). These two effects are reminiscent of what is observed when recycling through this pathway is blocked, either via inhibition of Arf6 (using dominant negative Arf6) or actin depolymerization (using cytochalasin D) (Radhakrishna and Donaldson, 1997). To further explore whether KSR1 overexpression perturbs trafficking through this pathway, the localization of additional markers was examined. In addition to Arf6 itself, we monitored TRE17(long), which we reported previously to traffic between the plasma membrane and tubular endosome (Martinu *et al.*, 2004). HeLa cells were transfected with HA-tagged forms of these proteins, either alone or together with FLAG-KSR1. As shown previously (D'Souza-Schorey *et al.*, 1997, 1998a; Radhakrishna and Donaldson, 1997; Radhakrishna *et al.*, 1999; Martinu *et al.*, 2004), both Arf6 and TRE17(long) resided predominantly at the plasma membrane when expressed by themselves (Figure 2, A and B). However, upon coexpression with FLAG-KSR1, plasma membrane staining was significantly reduced, and Arf6 and TRE17(long) accumulated at the numerous tubular elements (Figure 2, C and D). Tubular retention of Arf6 and TRE17(long) was observed in all cells coexpressing KSR1. These results suggest that KSR1's effects are not specific for MHCI. Rather, KSR1 overexpression perturbs the subcellular distribution of other markers of the Arf6 pathway, inducing intracellular accumulation at the expanded endosomal compartment at the expense of plasma membrane localization.

KSR1 Induces Expansion of the Tubular Endosomal Compartment in a Manner That Correlates with Inhibition of Erk

Scaffold proteins promote signaling by facilitating interaction between components of a signaling module (Morrison and Davis, 2003). However, their effects are dose dependent, because scaffold overexpression often causes segregation of components into distinct, nonfunctional complexes. Indeed, overexpression of KSR1 has been shown to inhibit Erk activation (Sundaram and Han, 1995; Therrien *et al.*, 1995, 1996; Michaud *et al.*, 1997; Denouel-Galy *et al.*, 1998; Joneson *et al.*, 1998; Cacace *et al.*, 1999; Brennan *et al.*, 2002; Nguyen *et al.*, 2002; Ohmachi *et al.*, 2002; Kortum and Lewis, 2004; Razidlo *et al.*, 2004). To gain insight into the mechanism by which KSR1 promotes expansion of the tubular endosomal compartment and alters the subcellular distribution of cargo, we

Figure 3. Expansion of the tubular endosome by KSR1 correlates with inhibition of Erk. (A) HA-Erk1 was cotransfected into HeLa cells with vector, FLAG-KSR1, or FLAG-KSR1(C809Y). Cells were starved overnight and then stimulated or not with EGF for 10 min. Cells extracts were subjected in immunoblotting using anti-HA and anti-FLAG antibodies. FLAG-KSR1 WT (B) or FLAG-KSR1(C809Y) (C) was transfected into HeLa cells and detected with anti-P392. Bar, 10 μ m.

examined its effects on Erk1 activity in our system. HeLa cells were cotransfected with FLAG-KSR1 and HA-Erk1, serum starved, and then stimulated with epidermal growth factor. Erk1 activation, indicated by a phosphorylation-induced decrease in its electrophoretic mobility, was monitored by immunoblotting with anti-HA. As seen in Figure 3A, activation of Erk1 was completely blocked by KSR1 coexpression. In contrast, a KSR1 point mutant defective in MEK binding (KSR1/C809Y) did not perturb activation of Erk1 (Figure 3A), consistent with previous reports (Muller *et al.*, 2000; Brennan *et al.*, 2002). Although KSR1/C809Y was able to localize to tubular elements, its overexpression failed to induce expansion of the compartment as WT KSR1 did (Figure 3, B and C). Thus, KSR1's effects on tubular endosome morphology correlate with its ability to inhibit Erk activation.

Erk and MEK Localize to the Tubular Endosome

One interpretation of the results mentioned above is that Erk activity is required to maintain normal membrane dynamics of the tubular endosome and/or for recycling from this compartment. It was therefore predicted that Erk1 and its upstream activating kinase MEK1 might reside at the tubular endosome. To examine this, HA-tagged forms of the proteins were transfected into HeLa cells. Although both HA-Erk1 and HA-MEK1 exhibited nondiffuse staining throughout the cytoplasm, distinct tubular localization was difficult to discern (Figure 4, A and B). However, in all cells coexpressing KSR1, tubular endosomal accumulation of Erk1 and MEK1 was readily apparent, as typified in Figure 4, C and D. We further confirmed that endogenous Erk and MEK were recruited to the tubular endosome. To facilitate their visualization, HeLa cells were transfected with FLAG-KSR1 to induce expansion of the tubular endosome and inhibit recycling. Cells were then analyzed by immunofluorescence microscopy, simultaneously probing with anti-P392 and anti-Erk or anti-MEK antibodies. As seen in Figure 4, E and F, both endogenous Erk and MEK localized to the tubular endosome. Endogenous KSR1 could not be visualized at this compartment, because anti-P392 antibodies were not sufficiently sensitive to detect native levels of the protein (our unpublished data).

Dominant Negative Erk Accumulates at the Tubular Endosome and Induces Its Expansion

The data mentioned above reveal a previously unrecognized subcellular compartmentalization for Erk, MEK, and KSR1. Furthermore, the expansion of the tubular endosome and the accumulation of cargo at this site upon Erk inhibition induced by KSR1 overexpression suggested a potential role for this signaling pathway in endosomal trafficking. To directly test whether Erk regulates dynamics of the Arf6 tubular endosome, we used a dominant negative allele of Erk2 (Erk2-AEF), which harbors substitutions in its two regulatory phosphorylation sites (Kato *et al.*, 1997). We reasoned that similarly to KSR1 overexpression, Erk2-AEF would inhibit Erk activation and thus perturb tubular endosome morphology. Supporting this notion, we observed that cells expressing Erk2-AEF exhibited a prominent tubular endosome (Figure 5A). Notably, Erk2-AEF was readily detected at the tubules even in the absence of KSR1 coexpression, in contrast to Erk2 wild type (WT) (Figure 5B). These results suggest that inhibition of Erk perturbs tubular endosome dynamics, leading to expansion of the compartment and accumulation of Erk itself at this site. Importantly, they also confirm that Erk is not merely recruited to the tubular endosome by overexpression of KSR1 in the experiments mentioned above but that it can localize there in the presence of endogenous KSR levels when inactive.

Pharmacological Inhibition of Endogenous Erk Perturbs the Subcellular Distribution of Arf6 Pathway Cargo

We next examined the role of endogenous Erk in regulating trafficking through the Arf6 pathway by analyzing the effects of U0126, a highly specific MEK inhibitor. Anti-MHCI antibody was added to the medium of live HeLa cells and allowed to internalize for 4 h, in the presence of U0126 or vehicle. Cells were then fixed and analyzed by confocal microscopy. In vehicle-treated control cells, the bulk of MHCI was detected at the cell surface (Figure 6, A and B), consistent with previous reports (Radhakrishna and Donaldson, 1997; Donaldson and Radhakrishna, 2001). In contrast, intracellular staining of MHCI on vesicles was significantly enhanced in cells treated with U0126 (Figure 6, A and C; additional wide-field images in Supplemental Figure 1). Transfection of cells with GFP-GFP-PLC δ (PH) aided in visualizing the association of the MHCI vesicles with the tubular endosome (Figure 6C). Immunoblotting confirmed that U0126 did not increase total levels of MHCI protein (Figure 6A). Importantly, the effects of U0126 were highly specific for Arf6 pathway cargo, because no alterations in the subcellular distribution of transferrin were discerned (Figure 6, D and E).

To quantify the effects of U0126 on the subcellular distribution of MHCI, flow cytometry was performed. HeLa cells were treated with vehicle or U0126 for 5 h, and the population of MHCI at the cell surface was detected by flow cytometry using FITC-conjugated anti-MHCI. U0126 caused a statistically significant reduction in the relative fraction of MHCI at the cell surface was observed (Figure 7A). Similar results were obtained after 1 h of U0126 treatment (our unpublished data). Furthermore, treatment of cells with the structurally unrelated MEK inhibitor PD98059 for 5 h reduced surface expression of MHCI to a similar degree (Figure 7A). Immunoblotting with antibodies that specifically recognize the active, phosphorylated form of Erk confirmed that both drugs effectively inhibited Erk1 and Erk2 (Figure 7A, inset). In contrast to MHCI, the subcellular distribution of transferrin was unaffected by Erk inhibition (Figure 7B).

Figure 4. Erk1 and MEK1 localize to the tubular endosome. HeLa cells were cotransfected with HA-Erk1 and GFP-PLCδ(PH) (A), HA-MEK1 and GFP-PLCδ(PH) (B), HA-Erk1 and FLAG-KSR1 (C), or HA-MEK1 and FLAG-KSR1 (D). Cells were subjected to indirect immunofluorescence using anti-HA or anti-P392 as indicated. (E and F) HeLa cells were transfected with FLAG-KSR1 and then probed with anti-P392 and anti-Erk (E) or anti-MEK (F). Bar, 10 μm.

As an independent means to verify the role of Erk in MHCII trafficking, small interfering RNA was used. However, consistent with previous studies knockdown of Erk1 and Erk2 expression was incomplete (Gaben *et al.*, 2004; Yun *et al.*,

2005); consequently, no effect on MHCII distribution was observed (our unpublished data). Nevertheless, our data using two independent MEK inhibitors strongly suggests that endogenous Erk activity is required for maintaining

Figure 5. Dominant negative Erk exhibits enhanced localization to the tubular endosome and induces its expansion. HeLa cells were transfected with FLAG-Erk2-AEF (A) or FLAG-Erk2 WT (B). Samples were probed with anti-Erk antibody. At the confocal laser settings used in this experiment, only transfected Erk2 and not endogenous Erk could be detected. Bar, 10 μ m.

normal trafficking through the Arf6 pathway and for controlling the subcellular distribution of its cargo.

U0126 Does Not Perturb the Kinetics of MHCI Endocytosis

The data mentioned above indicate that inhibition of Erk reduces cell surface levels of MHCI, concomitant with an increase in intracellular levels. This could arise either through an enhanced rate of endocytosis or a reduced rate of recycling. To distinguish between these possibilities, initial endocytosis rates were measured. HeLa cells were pretreated with U0126 or vehicle for 5 h and then incubated on ice with anti-MHCI antibody to label the surface population of MHCI. Cells were transferred to 37°C for various times to allow internalization. The fraction of anti-MHCI remaining at the cell surface at each time point was monitored using fluorescently conjugated secondary antibody and then quantified by flow cytometry. In control cells, MHCI was rapidly endocytosed within 20 min (Figure 7C). U0126 had no effect on the rate of MHCI internalization (Figure 7C). Endocytosis rates were similarly unaffected when cells were pretreated with the drug for 1 h (our unpublished data). The fact that the internalization rates remained unaffected with either time of U0126 pretreatment, but steady-state surface levels of MHCI were reduced (Figure 7A) strongly suggests that U0126 exerts its effects by inhibiting the recycling of MHCI to the plasma membrane.

Time-Lapse Imaging of U0126 Effects on Tubular Endosome Morphology and Dynamics

To gain further insight into the mechanism by which Erk regulates recycling through the Arf6 pathway, we monitored the effects of U0126 on tubular endosomal dynamics in living cells. Previous work has shown that the tubular elements are dynamic, often extending, retracting, and moving through the cytoplasm (Brown *et al.*, 2001; Weigert *et al.*, 2004). To visualize this compartment, HeLa cells were transfected with GFP-PLC δ (PH), and images were collected every 30 s for 1 h. In cells treated with vehicle, tubules were found to be dynamic as reported previously. However no significant or persistent change in the number of tubules or the intensity of GFP-PLC δ (PH) labeling of the tubules was observed during the period of observation (Figure 8A). In contrast, addition of U0126 led to a rapid increase in the number of tubules per cell and in the intensity of GFP-PLC δ (PH) labeling of individual tubules (Figure 8, B and C). We simultaneously monitored the effects of Erk inhibition on the dynamics of transferrin trafficking. HeLa cells were transfected with GFP-PLC δ (PH), and Alexa Fluor 546-labeled transferrin was added to the culture medium in the

presence of U0126. Although the GFP-PLC δ (PH)-positive tubules increased in number and intensity, in the same cells no significant change was discerned in Alexa Fluor 546-transferrin staining, nor was any morphological alteration in the transferrin-positive structures observed (Figure 8, C and D). These data confirm a highly specific and rapid effect of Erk inhibition on the dynamics of the Arf6 recycling compartment, which ultimately leads to reduced recycling and diminished steady-state levels of MHCI at the cell surface.

Epistasis Analysis of Erk and Arf6

We next explored the epistatic relationship between Erk and Arf6 in recycling through this pathway. Our data thus far are consistent with Erk functioning at the tubular endosome, perhaps in promoting budding of vesicles/transport carriers. The precise stage(s) at which Arf6 functions in recycling is unclear, although several studies suggest that it is required for the latest steps of secretion (Vitale *et al.*, 2002a,b; Prigent *et al.*, 2003). Indeed, the exocyst, a large protein complex that tethers secretory vesicles to the plasma membrane, was recently identified as an Arf6 effector (Prigent *et al.*, 2003). Thus, Erk seems to function at an earlier step in recycling than Arf6. To test this, we examined the effect of U0126 on Arf6 activation. It has been proposed that Arf6 is in its inactive state at the tubular endosome (Peters *et al.*, 1995; Radhakrishna and Donaldson, 1997; D'Souza-Schorey *et al.*, 1998; Donaldson and Radhakrishna, 2001) and is predominantly activated upon delivery to the plasma membrane, where its guanine nucleotide exchange factors (GEFs) are recruited (Venkateswarlu *et al.*, 1998; Franco *et al.*, 1999; Langille *et al.*, 1999; Niedergang *et al.*, 2003). U0126 would be predicted to perturb this delivery and therefore inhibit Arf6 activation. HeLa cells were cotransfected with HA-Arf6 and a FLAG-tagged form of the Arf6 GEF, EFA6, or control vector. Cell lysates were subjected to pull-downs using GST-GGA3 beads, which specifically precipitate the GTP-bound form of Arf6 (Santy and Casanova, 2001; Martinu *et al.*, 2004). As seen in Figure 9A, EFA6 potently activated Arf6 (compare lanes 1 and 2 with 3 and 4). Treatment of cells with U0126 modestly, but reproducibly, inhibited EFA6-induced activation of Arf6 (Figure 9A). This partial effect is consistent with the decrease, but not elimination, in surface delivery of MHCI caused by U0126.

If Arf6 functions at a later step than Erk, then Erk would be predicted to be sequestered in recycling vesicles that express dominant negative Arf6 (Arf6 T27N), which blocks vesicle fusion with the plasma membrane. As shown in Figure 9B, Erk1 indeed accumulated on Arf6 T27N-induced vesicles and tubules. Stimulation with EGF or serum failed to rescue sequestration of Erk1 (our unpublished data). We further examined the impact of this sequestration on agonist-induced activation of Erk. HeLa cells were transfected with HA-Erk1 or FLAG-Erk2 in the absence or presence of Arf6 T27N, starved, and then stimulated with EGF for various times. Cell extracts were immunoblotted with anti-HA or anti-FLAG antibody to monitor phosphorylation-induced mobility shift of Erk. As seen in Figure 9C, Arf6 T27N partially but significantly inhibited EGF-induced activation of both Erk1 and Erk2. Anti-phosphotyrosine blotting confirmed that the EGF receptor was appropriately activated in all samples (Figure 9C). Conversely, coexpression of constitutively active Arf6 (Arf6 Q67L) increased basal activity of endogenous Erk1 and Erk2, as revealed by anti-phospho-Erk blotting (Figure 9D). We noted that endogenous Erk1 and Erk2 were also reproducibly activated upon expression of EFA6 (Figure 9A, anti-P-Erk). Together, these data reveal a complex epistatic relationship between Erk and Arf6;

Figure 6. Pharmacological inhibition of endogenous Erk induces intracellular accumulation of Arf6 pathway cargo. (A) HeLa cells were pretreated for 1 h with DMSO or U0126 (50 μ M) and then incubated with anti-MHCI for 4 h in the continued presence of the drug. Cells were fixed and processed for confocal microscopy. Parallel samples were subjected to immunoblotting with anti-MHCI to confirm that total MHC1 levels were not altered by the drug. (B–E) HeLa cells were transfected with GFP-PLC δ (PH) and pretreated for 1 h with vehicle (B and D) or U0126 (50 μ M) (C and E). Cells were then incubated with anti-MHCI (B and C) in the continued presence of drug for 4 h. Images in B and C were scanned using identical laser settings. (D and E) Cells were incubated with Alexa Fluor 546-conjugated-transferrin in the continued presence of drug for 1 h. Bar, 10 μ m.

whereas Erk seems to function at an earlier step of recycling than Arf6, Erk activation is also partially dependent on Arf6. In essence, Erk and Arf6 seem to have dual functions as both cargo and regulators of this pathway, and a reciprocal regulation thereby exists.

DISCUSSION

Our work uncovers a novel role for Erk in regulating organelle dynamics and endosomal trafficking. We show that

Erk, MEK, and KSR1 function specifically in the nonclathrin plasma membrane–endosomal recycling system regulated by Arf6 but that they have no discernible effect on trafficking through the classical clathrin-dependent pathway. Erk signaling is required to maintain normal morphology and dynamics of the Arf6 tubular endosome. Inhibition of Erk activity, either pharmacologically with U0126 treatment or “genetically” by overexpression of KSR1 or dominant negative Erk, induces dramatic alterations in its morphology, with tubules becoming more abundant. These perturbations

Figure 7. U0126 reduces surface expression of MHCI without affecting initial endocytosis rates. HeLa cells were incubated with U0126 (50 μ M), PD98059 (50 μ M), or vehicle (DMSO) for 5 h. Surface populations of MHCI (A) and transferrin receptor (TfR) (B) were quantified by flow cytometry as detailed in *Materials and Methods*. Graphs on left show cell surface expression relative to vehicle-treated cells; error bars indicate SE of the mean. Asterisks denote statistically significant reduction in surface staining of MHCI upon addition of U0126 and PD98059 treatment (p values of 0.006 and 0.005, respectively). Representative histograms are shown on right. Data represent results from three to eight independent experiments, each performed in duplicate. In A, anti-P-Erk immunoblots were performed to confirm efficient inhibition of Erk by the drugs. (C) HeLa cells were pre-treated with U0126 or DMSO for 5 h. Surface MHCI was labeled by incubation of cells with anti-MHCI antibody on ice for 1 h and then allowed to internalize by transfer to 37°C for the indicated times. For each time point, the population of anti-MHCI remaining at the cell surface was detected with FITC-conjugated secondary antibody, and monitored by flow cytometry. Results represent the data from four independent experiments, each performed in duplicate.

ultimately lead to reduced steady-state levels of Arf6 pathway cargo at the cell surface.

The mechanism by which inhibition of Erk signaling elicits these effects remains to be determined. U0126 did not affect initial endocytosis rates, suggesting that the recycling branch of the pathway is the target of its action. The major

effect of Erk inhibition is morphological alteration (i.e., expansion) of the tubular endosome; we speculate that this leads to reduced recycling from the compartment. Although U0126 did cause a statistically significant reduction in surface levels of MHCI, the inhibition was modest. In contrast, KSR1 overexpression induced dramatic tubular endosomal proliferation and significantly inhibited plasma membrane localization of various Arf6 pathway markers (Figures 1 and 2). Thus, at supraphysiological levels KSR1 may have effects in addition to Erk inhibition that alters tubular endosome dynamics.

Our studies support a recently described link between Arf6 and Erk. D'Souza-Schorey and colleagues reported that Arf6 Q67L induces Erk activation, whereas Arf6 T27N inhibits hepatocyte growth factor-induced activation (Tague *et al.*, 2004), although the mechanism by which Arf6 functions was not known. Our work extends these findings, showing that Arf6 activity is required for activation of Erk by EGF and that the Arf6 GEF EFA6 can also induce Erk activation. We further provide a possible mechanism by which Arf6 exerts its effects: Arf6 may regulate trafficking of the subpopulation of Erk, MEK, and KSR at the tubular endosome. Agonist stimulation, which enhances recycling through the Arf6 pathway (Powelka *et al.*, 2004), may promote delivery of the Erk/MEK/KSR complex to the plasma membrane, facilitating its interaction with Raf. Another possibility, which is not mutually exclusive, is that Arf6 may promote activation of Erk at the tubular endosome (i.e., independently of translocation to the plasma membrane). At first glance, this may seem to be at odds with data that suggests that Arf6-GTP is localized to the plasma membrane and that Arf6-GDP resides intracellularly on tubules and vesicles (Peters *et al.*, 1995; Radhakrishna and Donaldson, 1997; D'Souza-Schorey *et al.*, 1998b; Donaldson and Radhakrishna, 2001). However, Arf6 regulation is likely more complex *in vivo*. Indeed, the tubular endosome is enriched in PI(4,5)P₂, the product of phosphatidylinositol 4-phosphate 5-kinase (PI4P5K), a GTP-dependent effector of Arf6 (Honda *et al.*, 1999; Brown *et al.*, 2001). Furthermore, Arf6-GDP has been detected at the plasma membrane (Macia *et al.*, 2004). Thus, although the GTP- and GDP-bound forms of Arf6 may be enriched at the plasma membrane and tubular endosome, respectively, Arf6 likely undergoes activation and inactivation at both locales.

Aside from Arf6, relatively little is known of the molecules that regulate trafficking through this pathway or that govern formation of the tubular endosome. Two proteins that have been implicated in these processes are Rab22a and EHD1 (Caplan *et al.*, 2002; Naslavsky *et al.*, 2004a; Weigert *et al.*, 2004). EHD1 is a member of the Eps15 homology (EH) domain-containing family of EHD proteins (Mintz *et al.*, 1999; Lin *et al.*, 2001; Caplan *et al.*, 2002). Dominant negative Rab22a, or RNA interference-mediated knockdown of EHD1 or Rab22a, inhibits tubule formation and recycling of MHCI (Caplan *et al.*, 2002; Weigert *et al.*, 2004). Conversely, overexpression of EHD1 stimulates MHCI recycling. Interestingly, cells expressing a constitutively active, GTPase-deficient mutant of Rab22a (Rab22aQ64L) exhibit a prominent tubular endosomal compartment and defective recycling of MHCI (Weigert *et al.*, 2004), reminiscent of the effects of Erk inhibition. Rab22aQ64L also causes the accumulation of enlarged vesicles at the cell periphery, suggesting that GTP hydrolysis by Rab22a may also be required for fusion of postendocytic vesicles with the plasma membrane (Weigert *et al.*, 2004). The precise mechanism by which EHD1 or Rab22a regulates tubular endosome formation and dynamics remains unknown. It will be of interest to determine

Figure 8. Time-lapse imaging of U0126 effects on tubular endosomal morphology. GFP-PLC δ (PH) was transfected into HeLa cells to visualize the tubular endosome. Cells were treated with vehicle (A) or 50 μ M U0126 (B–D), and images were collected every 30 s for 1 h. Images from 0-, 20-, 40-, and 60-min time points are shown. In C and D, HeLa cells transfected with GFP-PLC δ (PH) were incubated with Alexa Fluor 546-transferrin and then monitored for 1 h in the presence of U0126. The GFP-PLC δ (PH) (C) and corresponding Alexa Fluor 546-transferrin staining (D) from the same cell group is shown. Results are representative of three independent experiments.

whether the activity of these proteins, either directly or indirectly, is modulated by Erk-mediated phosphorylation.

Alternative mechanisms by which Erk might regulate tubular endosomal dynamics may be speculated. Time-lapse imaging of U0126-treated cells revealed a rapid and time-dependent increase in the intensity of GFP-PLC δ (PH) labeling of the tubules. Because this construct recognizes PI(4,5)P₂, one interpretation of this result is that Erk may regulate lipid metabolism at the tubular endosome. Indeed, previous work from Donaldson and coworkers has shown that sustained activation of PI4P5K profoundly disrupts tubular endosome morphology (Brown *et al.*, 2001). Another possibility is that Erk regulates membrane dynamics of the compartment and that the increased labeling of GFP-PLC δ (PH) merely reflects its expansion. For example, Erk may normally promote budding of transport carriers from

the tubular endosome; U0126 would perturb the equilibrium between vesicle fission and fusion that normally maintains the size of this compartment and lead to its expansion. Yet another possibility is that Erk may modulate the activity of proteins that promote tubulation of membranes, such as Bin-Amphiphysin-Rvs domain-containing proteins (Peter *et al.*, 2004; Zimmerberg and McLaughlin, 2004; Gallop and McMahon, 2005). Exploring these various possibilities will be the goal of future investigation.

Our studies highlight the diversity of Erk's cellular functions. Erk is one of the most abundant kinases in the cell and functions in gene expression, vesicle trafficking, cell adhesion, and motility (Klemke *et al.*, 1997; Torii *et al.*, 2004b; Viala and Pouyssegur, 2004). Erk must therefore be exquisitely regulated both temporally and spatially to mediate the phosphorylation of specific substrates at distinct subcellular

Figure 9. Epistasis analysis of Arf6 and Erk. (A) HeLa cells were transfected with HA-Arf6 together with FLAG-EFA6 or control vector and then treated with U0126 (+) or vehicle (–) for 5 h. Cell extracts were pulled down with GST-GGA3 beads and then subjected to anti-HA immunoblotting to detect active Arf6 (top). Whole cell lysates (WCL) were also subjected to immunoblotting with the indicated antibodies; EFA6 was detected with anti-FLAG. Data represent the results of three independent experiments. (B) HeLa cells were cotransfected with HA-Arf6 T27N (green) and Myc-Erk1 (red), and visualized by immunofluorescence confocal microscopy. (C) HeLa cells were cotransfected with HA-Erk1 or FLAG-Erk2, together with Arf6 T27N (+) or control vector (–), serum starved, and then stimulated with EGF (100 ng/ml) for the indicated times. WCL were immunoblotted with anti-HA, anti-FLAG, anti-Arf6, and anti-phosphotyrosine (PY) antibodies (left). (D) HeLa cells were transfected with Arf6 Q67L or vector and then immunoblotted with the indicated antibodies.

locations (Morrison and Davis, 2003; Yoshioka, 2004). As mentioned above, Erk is localized to the Golgi by Sef and to late endosomes by MP-1. Together with our results indicating that KSR1 can recruit Erk to the tubular endosome, this suggests that Erk is recruited to distinct endosomal compartments by distinct scaffolds. In addition to these membranous organelles, activation of Erk is mediated at focal adhesions by the GIT1 scaffold (Yin *et al.*, 2004). Erk signaling promotes the turnover of focal adhesions, although the identity of its relevant substrates is not fully understood. Finally, the most recently identified Erk scaffold is IQGAP1 (Roy *et al.*, 2004), an actin-binding protein and effector of Rho family GTPases (Briggs and Sacks, 2003). IQGAP1 localizes to the leading edge of motile cells (Nabeshima *et al.*, 2002; Mataraza *et al.*, 2003; Watanabe *et al.*, 2004), and it may aid in restricting Erk activation at this site to promote directed migration. Together, these studies underscore the diversity of scaffolds that direct Erk signaling in a highly localized manner for distinct cellular processes.

Our work describes a previously unappreciated site of action for KSR1. Previous work has shown that KSR1 shuttles between the cytosol and plasma membrane in a manner regulated by phosphorylation of S392. Phospho-S392 mediates binding to 14-3-3, which leads to cytosolic retention of KSR1 presumably by masking its plasma membrane-interacting CA3 domain (Muller *et al.*, 2001; Zhou *et al.*, 2002; Ory *et al.*, 2003). On mitogenic stimulation, this site is dephosphorylated, allowing translocation of KSR1 to the plasma membrane (Muller *et al.*, 2001; Ory *et al.*, 2003). Consistent with this model, mutation of S392 to alanine results in constitutive localization of KSR1 to the plasma membrane (Muller *et al.*, 2001). Thus, phosphorylation of S392 has typically been associated with the inactive state of KSR1. Notably, immunolocalization of KSR1 using anti-P392 has not been previously reported. We have found a striking enrichment of KSR1 phosphorylated on serine 392 (P392-KSR1) at the tubular endosome. The mechanism of its recruitment to the tubular endosome remains unknown.

In conclusion, this study identifies a novel role for Erk signaling in regulating clathrin-independent endosomal trafficking through the Arf6 pathway. Recycling through this pathway has been implicated in a variety of functions, including cell spreading, lamellipodia formation, motility, and phagocytosis (Song *et al.*, 1998; Zhang *et al.*, 1998; Radhakrishna *et al.*, 1999; Donaldson, 2003; Niedergang *et al.*, 2003). Our experiments therefore introduce a possible novel means by which Erk may participate in the control of these fundamental cellular processes.

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