Late Endosomal Traffic of the Epidermal Growth Factor Receptor Ensures Spatial and Temporal Fidelity of Mitogen-activated Protein Kinase Signaling[□]

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Mitogen-activated protein kinase (MAPK) signaling is regulated by assembling distinct scaffold complexes at the plasma membrane and on endosomes. Thus, spatial resolution might be critical to determine signaling specificity. Therefore, we investigated whether epidermal growth factor receptor (EGFR) traffic through the endosomal system provides spatial information for MAPK signaling. To mislocalize late endosomes to the cell periphery we used the dynein subunit p50 dynamitin. The peripheral translocation of late endosomes resulted in a prolonged EGFR activation on late endosomes and a slow down in EGFR degradation. Continuous EGFR signaling from late endosomes caused sustained extracellular signal-regulated kinase and p38 signaling and resulted in hyperactivation of nuclear targets, such as Elk-1. In contrast, clustering late endosomes in the perinuclear region by expression of dominant active Rab7 delayed the entry of the EGFR into late endosomes, which caused a delay in EGFR degradation and a sustained MAPK signaling. Surprisingly, the activation of nuclear targets was reduced. Thus, we conclude that appropriate trafficking of the activated EGFR through endosomes controls the spatial and temporal regulation of MAPK signaling.

INTRODUCTION

Signal transduction and endocytosis are inseparably linked cell biological functions. Cells are constantly exposed to an extracellular environment that needs correct interpretation. Cell surface receptors are often used to receive this information and transduce it to intracellular signaling cascades. Yet, the tremendous amount of extracellular information is translated by a relatively limited repertoire of signaling molecules. Therefore, cells use sophisticated mechanisms to perform their function in a given biological context. Thus, signal transduction requires precise spatial and temporal regulation to define a unique biological response (Di Fiore and De Camilli, 2001; Seto *et al.*, 2002; Teis and Huber, 2003).

Receptor tyrosine kinases (RTKs) represent a large family of cell surface receptors. Ligand-induced activation of RTKs, including the epidermal growth factor (EGF) receptor (EGFR), results in signaling and in a rapid and efficient internalization (Haigler *et al.*, 1978). Internalized activated cell surface receptors can either be recycled to the plasma membrane (PM) or

sorted from early endosomes to the late endosomal compartment from where they are transported to lysosomes for degradation (Raiborg *et al.*, 2002; Sachse *et al.*, 2002).

Mitogen-activated protein kinase (MAPK) pathways are frequently coupled to various extracellular signals from RTKs. MAPK modules are part of a complex network of signaling cascades. To generate an appropriate output signal, the coordination of input pathways and subsequent integration of the according signals are necessary. Therefore, MAPK modules have evolved strategies for molecular signal interpretation. One strategy to achieve this specificity is to organize multiprotein complexes (Pawson and Scott, 1997). Scaffold proteins are used to organize specific signaling units. Additionally, adaptors localize scaffold complexes to distinct subcellular compartments to ensure specificity and spatial as well as temporal regulation of signal transduction (Kolch, 2000).

Crucial signaling components of the MAPK signaling pathway are found at the PM and on endosomes. Additionally, RTKs transmit signals from endosomes because the internalized EGFR signals until it is finally taken up in lysosomes and degraded (Burke *et al.*, 2001). Furthermore, it has been demonstrated that activated MAPKs localize to endosomal membranes, thereby creating signaling endosomes (Howe *et al.*, 2001; Teis *et al.*, 2002). We have recently shown that the endosomal p14-MP1 scaffold complex recruits mitogen-activated protein kinase kinase (MEK)1 to regulate the subcellular position and traffic of late endosomes which controls MAPK signaling specificity (Teis *et al.*, 2006). Perturbation of endosomal p14-MP1-MEK1 signaling is detrimental for tissue homeostasis, and a mutation in human p14 causes a primary immunodeficiency syndrome (Teis *et al.*, 2006; Bohn *et al.*, 2007). Endocytosis and con-

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Abbreviations used: EEA1, early endosomal antigen 1; HRP, horseradish peroxidase; LAMP1, lysosomal-associated membrane protein 1; LBPA, lysobiphosphatic acid; MVB, multivesicular body; p-ERK, phospho-extracellular signal-regulated kinase; p-p38, phospho-p38.

trolled endocytic traffic are required to regulate signal duration. These findings support the concept that the endosomal system serves as an intracellular site for the regulation of signal transduction (Cavalli *et al.*, 2001; Di Fiore and De Camilli, 2001).

Thus, we propose that regulated transport of activated cell surface receptors through endosomes provides sufficient spatial resolution to shape signaling specificity. To test this hypothesis, we changed the position of endosomes relative to the nucleus, and we determined the effects on EGFR and MAPK signaling.

The steady-state localization of endomembrane systems such as the endoplasmic reticulum, Golgi apparatus, endosomes, and lysosomes require both intact microtubules and the activities of microtubule-based motor proteins (Goodson *et al.*, 1997). Cytoplasmic dynein, the predominant cytosolic minus end-directed motor, in conjunction with its activator dynactin (Gill *et al.*, 1991), keeps the Golgi complex, endosomes, and lysosomes in their normal juxtanuclear position (Burkhardt *et al.*, 1997; Harada *et al.*, 1998).

We used the dynein subunit p50 dynamitin to manipulate the position of late endosomes. Dynactin functions as an adaptor that allows dynein to bind cargo (Quintyne *et al.*, 1999). Overexpression of dynamitin disrupts the dynactin complex and inhibits dynein-driven events of mitosis and organelle transport (Echeverri *et al.*, 1996; Burkhardt *et al.*, 1997; Wang *et al.*, 2003) including that of late endosomes and lysosomes (Valetti *et al.*, 1999; Jordens *et al.*, 2001). This results in an accumulation of the late endosomal compartment at the cell periphery. Additionally, we used the dominant-active version of Rab7, EGFP-Rab7Q67L, as an opposed model, because its expression clusters tightly the late endosomal compartment in the perinuclear region (Bucci *et al.*, 2000). These represent two contrasting model situations that we took advantage of to address whether an altered spatial distribution of the late endosomal compartment influences RTK transport and subsequently the cytoplasmic and the nuclear activation patterns of MAPK signaling pathways.

In this study, we demonstrate that the localization of the late endosomes influences the duration of the EGFR signaling, which in turn is important for the regulation of the signaling quality of MAP kinases. Mislocalization of the late endosomes to the cell periphery results in a sustained activation of phosphorylated (p)-extracellular signal-regulated kinase (ERK) and p-p38. Additionally, the prolongation of the EGFR signaling causes a stronger activation of the nuclear transcription factor Elk-1. In contrast, the clustering of the late endosomes in the perinuclear region results in a sustained activation of p-ERK but not of p-p38, which in turn decreases Elk-1 activation.

MATERIALS AND METHODS

Antibodies, Reagents, and Constructs

The phospho-ERK1/2, phospho-p38, and ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). The human CD107a (lysosomal associated membrane protein 1 [LAMP]1) antibody was obtained from BD Biosciences Pharmingen (San Diego, CA), and the early endosomal antigen (EEA)1 antibody from BD Biosciences (San Jose, CA). The lysobiphosphoric acid (LBPA) antibody was a generous gift from Jean Gruenberg (Department of Biochemistry, University of Geneva, Geneva, Switzerland). α -Tubulin antibody and EGF were purchased from Sigma-Aldrich (St. Louis, MO). LysoTracker DND-99 and secondary antibodies (Alexa488, Alexa568, and Alexa647) and the Alexa568-labeled EGF were obtained from Invitrogen (Carlsbad, CA). The EGFR antibody and the phospho-EGFR antibody were purchased from Chemicon International (Temecula, CA). The EGFR antibody for immunoblots was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The hemagglutinin (HA) antibody was a gift from Peter van der Sluijs (Department of Cell Biology, Utrecht University, 3584 CX Utrecht, The Netherlands).

Cell Culture, Transfection, and Constructs

HeLa cells were grown in high-glucose DMEM (Invitrogen) supplemented with 50 IU/ml penicillin, 50/ml streptomycin, and 10% fetal calf serum (Invitrogen) at 37°C, in 5% CO₂ and 98% humidity. Media and reagents for tissue culture were purchased from Invitrogen. The stable PathDetect Trans-Reporter cell line (Stratagene, La Jolla, CA) was grown according to the manufacturer's instructions. Cells were transfected with various constructs using Lipofectamine Plus (Invitrogen) following the manufacturer's suggestions. The construct for active Rab7 mutant-EGFP fusion protein (EGFP-Rab7Q67L, referred to as Rab7da) was a generous gift from Cecilia Bucci (Department of Clinical and Experimental Medicine, Federico II, Napoli, Italy) (Bucci *et al.*, 2000). The green fluorescent protein (GFP)-p50 (Quintyne *et al.*, 1999) and the HA-p50 (Valetti *et al.*, 1999) constructs were a generous gift from Trina A. Schroer (Department of Biology, Johns Hopkins University, Baltimore, MD 21218).

Immunoblots

Transfected or control cells were starved (14 h) and stimulated with 100 ng/ml EGF for the indicated times. The internalization of EGF was stopped by washing twice with ice-cold phosphate-buffered saline (PBS). Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM Na₃VO₄, 50 mM NaF, and proteinase inhibitors [aprotinin, pepstatin, leupeptin, and Pefabloc SC (Fluka, Biochemika)]), lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted, and probed with the respective antibodies.

EGFR degradation curves were performed as follows. The same protein amount of all samples was loaded, ERK and EGFR levels were calculated by Alpha EaseFC Software, version 4 (Alpha Innotech, San Leandro, CA). EGFR levels were adjusted to the corresponding ERK levels. Finally, EGFR levels were normalized to the EGFR levels of the control cells at time point zero.

Luciferase Assay

The luciferase assays were performed using the stable PathDetect Trans-Reporter cell lines (Stratagene) according to the manufacturer's instructions. After transfection, cells were serum-starved for 14 h in DMEM, and then they were stimulated for 0, 20, and 120 min with DMEM containing 100 ng/ml EGF. After the stimulation, the EGF containing DMEM was washed away twice with DMEM. Cells were harvested and lysed in luciferase lysis buffer (20 mM glycyl-glycine, pH 7.8, 50 mM NaCl, 2 mM EDTA, 1 mM MgSO₄, 5 mM dithiothreitol, 1% Triton X-100, and proteinase inhibitors [aprotinin, pepstatin, leupeptin, and Pefabloc SC]) 5 h after stimulation. One aliquot of the cell extract was used to measure the luciferase activity. The other aliquot was used to control protein expression and total protein amounts by immunoblotting.

Immunofluorescence

Pulse-chase experiments were performed as described previously (Petiot *et al.*, 2003). HeLa cells were either left untreated or stimulated with 100 ng/ml EGF after starvation for 14 h. Then, they were preincubated with DMEM containing Alexa568-labeled EGF for 1 h at 4°C to allow the EGF bind to the EGFR. Cells were stimulated for 10 min at 37°C (pulse) to ensure a simultaneous internalization of the EGF, followed by a 0-, 10-, 20-, 60-, and 120-min chase in DMEM in the absence of EGF. The endosomal trafficking of the EGF was stopped by washing the cells with ice-cold PBS buffer. Cells were immediately fixed on ice for 10 min with ice-cold 4% paraformaldehyde supplemented with 2 mM Na₃VO₄ in cytoskeleton buffer (CB) [10 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM MgCl₂]. Next, cells were permeabilized in 0.2% Triton X-100 in CB for 2 min. After 30 minutes of blocking in gelatin containing blocking buffer, cells were incubated for 1.5 h with the primary antibody, washed five times in CB and 50 mM NH₄Cl, incubated for 40 min with the secondary (Alexa-labeled) antibody, washed five times in CB and 50 mM NH₄Cl, and mounted in Mowiol (Polyscience, Warrington, PA). Confocal image analysis was performed using an LSM 510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY). The immunofluorescence staining for LBPA was performed as described previously (Kobayashi *et al.*, 1999).

In addition to Zeiss software, images have been converted to Photoshop, version 9.0 (Adobe Systems, Mountain View, CA). Brightness, contrast, or tonal value was improved, and figures were arranged with Macromedia FreeHand 10 or MX 11.0 software (Adobe Systems) and exported as *.jpeg files.

Immunoelectron Microscopy (IEM) and Cryo-Electron Microscopy (EM)

HeLa cells were starved and stimulated for 60 or 120 min with EGF either continuously or as pulse-chase experiment as described above (both kinds of experiments yielded essentially similar results as to the ultrastructural localization of the EGFR at these time points). Stimulated cells were immediately fixed with 4% (wt/vol) formaldehyde (freshly made from paraformaldehyde) in 0.1 M phosphate buffer (2 h at room temperature), and they were processed for Tokuyasu-immunolabeling (Liou *et al.*, 1996). In brief, thawed, ultrathin

Figure 1. The late endosomal compartment is mislocalized to the cell periphery in p50-overexpressing cells. (A) Distribution of the endosomal system in normal (left) and in p50-overexpressing cells (right). (B) Confocal images of GFP-p50 (green)-expressing cells and control cells. Top, indirect immunofluorescence analysis was performed using LAMP1 antibody (red). Bottom, incubation with LysoTracker (red) for 30 min. Arrows indicate the peripheral distribution of late endosomes/lysosomes in p50-expressing cells. Triangles indicate the perinuclear distribution in control cells. Bars, 10 μ m. (C) Demonstration of the distribution of late endosomes in control and GFP-p50-expressing cells. Black bars represent peripheral distribution, and white bars represent perinuclear distribution of LAMP1-positive structures; 92.3% exhibited peripheral and 7.7% perinuclear distribution in GFP-p50 cells versus 0.3% exhibited peripheral and 99.7% perinuclear distribution of LAMP1-positive structures in control cells. Mean $\pm$ SD of three independent experiments is indicated. (D) Peripherally mislocalized LAMP1- and EEA1-positive compartments represent different subpopulations of organelles. Confocal images of control cells and HeLa cells expressing GFP-p50 (green) are shown. Cells were subjected to indirect immunofluorescence analysis by using anti-EEA1 (red) and anti-LAMP1 (blue) antibody. High-magnification views are shown in the rectangles. Bar, 10 μ m.

cryosections were single-, double-, or triple-labeled, and bound antibodies were visualized by relevant secondary antibodies conjugated to colloidal gold. Antibodies used here included sheep anti-EGFR (Fitzgerald Industries, Concord, MA; see White *et al.*, 2006), mouse anti-LAMP1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit anti-GFP (Abcam, Cambridge, MA; see Stang *et al.*, 2004), and mouse anti-HA (from Peter van der Sluijs); furthermore, gold-conjugates from British Biocell (Cardiff, United Kingdom) (donkey anti-sheep 5 nm, rabbit anti-mouse 10 nm, and goat anti-rabbit 20 nm). The IEM data presented here are obtained from four series of transfection and EGF-stimulation experiments (i.e., for GFP-p50 and HA-p50, at least 3 independent series of each experiment; for GFP-Rab7da, 2 independent transfections). Immunogold labeling experiments were repeated twice with each sample. Sections were analyzed with a Philips CM120 (FEI, Eindhoven, The Netherlands), and images were recorded with a MORADA digital camera (Olympus SIS, Münster, Germany) or with negative film. Contrast and brightness of the digital images were optimized by using gray scale modification and high-pass filtering with Adobe Photoshop software, version 9.

High-pressure freezing was used instead of chemical fixation for complementing the morphological characterization of Rab7da- and p50-overexpressing cells. In brief, cells were cultivated on carbon-coated sapphire coverslips, transfected, stimulated, and subjected to high-pressure freezing followed by freeze-substitution and epoxy resin embedding (Hess *et al.*, 2000; Haller *et al.*, 2001).

Terminological Remark

We do not distinguish here between different subpopulations of late endosomal compartments or multivesicular bodies (MVBs), which is out of the scope of the present article (for discussion of this topic, see Geuze, 1998; Mobius *et al.*, 2003; Gruenberg and Stenmark, 2004; White *et al.*, 2006).

RESULTS

GFP-p50 Overexpression Mislocalizes the Late Endosomal Compartment to the Cell Periphery

To determine how the subcellular distribution of late endosomes impacts on MAPK signaling, we manipulated the position of the late endosomal compartment and shifted it from the perinuclear region (Figure 1A, left) to the cell

periphery (Figure 1A, right). For this purpose, the dynein subunit p50 dynamitin was used. Immunofluorescence analysis was used to study the distribution of endosomes in p50-expressing cells. Upon overexpression of GFP-p50 in HeLa cells, LAMP1-positive compartments (late endosomes, lysosomes) accumulate at the cell periphery (Figure 1B, top, arrow), whereas control cells (untransfected cells) show a typical perinuclear distribution (Figure 1B, top, triangle). Labeling of GFP-p50-expressing cells with the fluorescent acidotrophic probe LysoTracker DND-99 revealed that the peripherally mislocalized endosomes conserved lysosomal features, such as acidification (Figure 1B, bottom, arrow). In contrast, control cells exhibited a perinuclear distribution of lysosomes (Figure 1B, bottom, triangle). Next, the peripheral and perinuclear distribution of LAMP1-positive structures was analyzed in GFP-p50-overexpressing and in control cells, respectively. The results of three independent experiments (Figure 1C) point out the intense mislocalization of LAMP1-positive structures in p50-overexpressing cells. Here, 92.3% of GFP-p50-positive cells exhibited peripheral (black bars) and only 7.7% perinuclear (white bars) distribution of late endosomes/lysosomes, whereas 0.3% of control cells exhibited peripheral and 99.7% perinuclear distribution.

Immunofluorescence experiments of control and GFP-p50-overexpressing cells were performed using EEA1 (red) for early and LAMP1 (blue) for late endosomes/lysosomes (Figure 1D). Although there was a certain colocalization of these two markers in control and p50-expressing cells, the majority of the mislocalized endosomes at the cell periphery showed a different distribution of EEA1 and LAMP1-positive compartments (magnification in rectangle).

Therefore, p50-overexpressing cells represent an appropriate model for our further investigations on the influence of the spatial distribution of late endosomes on the kinetics of MAPK signaling pathways.

EGF Is Transported to Peripherally Mislocalized Late Endosomes in p50-expressing Cells

We characterized the internalization of the EGFR in GFP-p50-overexpressing HeLa cells to analyze whether the mislocalization of the late endosomal compartment interferes with cell surface receptor trafficking. For this purpose, internalization studies with Alexa568-labeled EGF were performed. Additionally, EGF transport in the cells was tracked by using markers for the different endosomal compartments: EEA1 (Supplemental Figure 1) for the early endosomes, LBPA (Supplemental Figure 2), and LAMP1 (Figure 2) for the late endosomes/lysosomes. This procedure allows following the EGF that has been internalized within the 10 min during the pulse. A minor redistribution of EEA1-positive compartments to the cell periphery could be observed in p50-overexpressing cells (Supplemental Figure 1). There were no significant alterations concerning EGF uptake and the early stages in the endocytic pathway like transport to the early endosomes in p50-expressing cells versus control cells. At later time points, high-magnification images show that there is still some colocalization of EEA1 and EGF; however, both in control and p50-expressing cells nearly to

the same extent. In general, the majority of the ligand was able to leave efficiently the early compartment after longer chase periods (Supplemental Figure 1).

Colocalization experiments of labeled EGF with LBPA (Supplemental Figure 2) and LAMP1 (Figure 2) were performed to visualize whether the mislocalized late endosomal compartment can be assessed by EGF. Unlike early endosomes, late endosomes undergo robust motility that is thought to use the dynein/dynactin motor complex, which results in a dramatic accumulation of the LBPA- and LAMP1-positive structures at the cell periphery in p50-overexpressing cells. There were no obvious differences in the EGF trafficking to the peripherally mislocalized LAMP1-positive structures in p50-expressing cells (Figure 2, high-magnification view in rectangle), even though this implied transportation to a completely different site in a cell. In control cells the EGF was detectable in the proximity of the nucleus. Interestingly, after a 60-min chase the majority of EGF in the control cells was mostly degraded, whereas in p50-expressing cells labeled EGF still colocalized with the dearranged LAMP1-positive compartments at the cell periphery (high magnification in rectangles). This phenomenon was even more obvious after the 120-min chase.

These internalization studies were repeated with an additional late endosomal/lysosomal marker LBPA (Supplemental Figure 2). The LBPA experiments confirmed our results obtained from previous experiments with LAMP1.

Figure 2. EGF is transported to peripherally mislocalized LAMP1-positive structures. HeLa cells expressing GFP-p50 were serum-deprived for 14 h. Cells were preincubated with DMEM containing Alexa586-labeled EGF (100 ng/ml) for 1 h at 4°C (referred to as 0' + 0'). Cells were stimulated for 10 min at 37°C (pulse) followed by a 0-, 10-, 20-, 60-, and 120-min chase in DMEM in the absence of EGF. Cells were subjected to indirect immunofluorescence analysis by using an antibody against LAMP1 and analyzed by triple channel fluorescence microscopy. p50-positive cells are shown in green, EGF in red, and LAMP1 in blue. Confocal images are shown. High-magnification views are shown in the rectangles. Bars, 10 μ m.

Figure 3. p50 overexpression results in a prolonged EGFR activation on peripherally mislocalized endosomes. (A) HeLa cells expressing GFP-p50 were pre-treated as described in Figure 2, and they were labeled with an antibody against the activated EGFR (blue). Confocal images are shown. High-magnification views are shown in the rectangles. Bars, 10 μ m. (B) The graph demonstrates the presence of the activated EGFR after 10 min of EGF pulse and 120 min of chase in GFP-p50-expressing HeLa cells (black) and control cells (white); 67% of GFP-p50-expressing cells exhibited activated EGFR after 120 min of chase, whereas only 20% in control cells; 100% are equivalent to 50 cells. Mean $\pm$ SD of three independent experiments is indicated.

p50 Overexpression Results in a Prolonged EGFR Activation on Peripherally Mislocalized Endosomes

Next, we addressed whether the EGFR was active on these peripherally mislocalized late endosomes in p50-overexpressing cells. Therefore, immunofluorescence pulse-chase experiments were performed with an antibody specific for the activated EGFR (Figure 3A). Cells that were incubated with EGF at 4°C displayed very little, if any, EGFR activation. On shift to 37°C, colocalization of the ligand EGF and the activated receptor was observed in GFP p50-expressing and in control cells. During longer chase periods, the activated EGFR in the control cells was internalized and transported to the perinuclear region where it was mostly degraded at 60 min (white circles) and undetectable at a chase

period of 120 min (white circles). In contrast, GFP-p50-overexpressing cells transported the activated EGFR to the peripherally mislocalized late endosomes (10- and 20-min chase), and they still showed EGFR activation after a 60- and 120-min chase (higher magnification in rectangle). Here, 67% of the GFP-p50-expressing cells exhibited activated EGFR after 120-min chase, whereas only 20% did in control cells (Figure 3B).

To further assess the defect in the endocytic EGFR traffic, immunoblot analyses were performed to investigate the EGFR degradation kinetics in p50-expressing cells versus control cells (Figures 4A and 9). In the control cells, the EGFR was degraded upon longer stimulation, as 60 or 120 min. However, in p50-expressing cells, the EGFR degrada-

tion was slowed down minor at 60 but more efficiently at 120 min of stimulation, which also resulted in a 2.4-fold up-regulation of EGFR levels at steady-state levels. The densitometric analysis of the normalized EGFR/ERK levels showed the slow down in the EGFR degradation in p50-expressing cells (Figure 9, black squares) compared with control cells (white squares). In the earlier time window between 0 and 20 min, the EGFR to ERK levels increase in the control cells, whereas they are already lowered in the p50-expressing cells. Thus, the levels of the p50-expressing cells (2.4-fold up-regulated) have been normalized to control levels (1-fold), the total EGFR/ERK levels are higher in p50-expressing cells. Despite the varying kinetics at earlier time points, the degradation rate between 20 and 120 min of stimulation is decreased in p50-expressing cells compared with control cells. However, p50 expression did not completely block EGFR degradation, because at later time points (240 min of EGF stimulation) EGFR levels decline gradually to zero (data not shown). Furthermore, the slow down of the EGFR degradation was dependent on the transfection efficiency of p50. Thus, the effect might be even stronger, because transfection efficiency of HA-p50 was only $\approx 70\%$.

The p50-induced peripheral translocation of the late endosomal compartment affected the degradation of the EGFR. It also resulted in a prolonged activation on peripherally mislocalized endosomes.

Spatial Distribution of EGFR Signaling Changes the Signaling Dynamics of MAPK Signaling Pathways

Next, we asked whether the mislocalization of the late endosomal compartment to the cell periphery also influences downstream MAPK signaling pathways. HA-p50-expressing HeLa cells and untransfected cells were starved for 14 h, and then they were stimulated with 100 ng/ml EGF in DMEM for 0, 10, 20, 60, and 120 min (Figure 4A). Immunoblot analysis revealed that p-ERK levels in control cells reached a maximum at 10 min. Interestingly, p50-expressing cells showed similar ERK activation at 10 and 20 min of EGF stimulation, but they exhibited a sustained p-ERK1/2 signaling throughout the whole stimulation period until 120 min. The activation of p38, another MAPK, was also changed in p50-expressing cells. In control cells, the activation of p38 was terminated after 20 min of EGF stimulation. However, in p50-expressing cells, the activation of p38 was

Figure 4. Spatial redistribution of EGFR signaling changes the activation pattern of MAPK signaling pathways. (A) p50 changes the activation pattern of cytoplasmic MAPK signaling targets. HeLa cells were transiently transfected with HA-p50 or left untreated. After 14-h serum deprivation, cells were stimulated with 100 ng/ml EGF for the indicated times. Cell lysates were separated by SDS-PAGE and probed with the indicated antibodies. The graphs show a densitometric analysis of the p-ERK1/2 and p-p38. One representative experiment of three is shown. (B) p50 overexpression influences nuclear targets. A stable Elk-1-driven luciferase reporter cell line was transfected with HA-p50 or with an empty vector. Cells were stimulated for the indicated times (0, 20, and 120 min) with EGF (100 ng/ml) or left untreated. One aliquot of the cell extract was used to measure the luciferase activity. The other aliquot was used to control protein expression and total protein amounts by immunoblotting. Each time point was performed twice and measured in triplicates. One representative experiment of three is shown. The relative light units are normalized to protein amount.

reduced at 10 and 20 min of stimulation, but remained sustained (Figure 4A).

Activated MAPK translocates into the nucleus to activate transcription factors such as Elk1 (Whitmarsh *et al.*, 1995). Thus, a stable Elk-1-driven luciferase reporter cell line was used to investigate if hyperactivation of ERK on peripheral endosomes translated into the nucleus (Figure 4B). Cells were stimulated for 20 and 120 min with 100 ng/ml EGF, or they were left untreated. The relative light units were normalized to the protein amount. Consistent with the sustained ERK activation, p50-expressing cells showed 3 times higher activation of the Elk-1 transcription factor upon EGF stimulation.

Spatial displacement of EGFR signaling resulted in a prolonged activation of cytoplasmic targets, e.g., p-ERK and p-p38. Additionally, the rearrangement of the late endosomal compartment also caused a major increase in the activation of the nuclear target Elk-1. Our data indicate that spatial information provided by the endosomal membrane system is an essential factor for the regulation of EGFR signaling and downstream signaling cascades.

GFP-Rab7da Expression Results in a Tight Clustering of Endosomes in the Perinuclear Region

We next asked whether perinuclear clustering of late endosomes would affect EGFR signaling in a way similar to the redistribution of the EGFR to the cell periphery. For this purpose, we used an active mutant of Rab7, EGFP-Rab7Q67L, referred to as Rab7da, as a complementary approach, which caused a tight clustering of the late endosomal structures in the perinuclear region (Figure 5A, right). To confirm that only the position of the late endosomal compartment was changed efficiently but that the distribution of the early endosomes remained unaffected, immunofluorescence analysis was performed (Figure 5B). Whereas the early endosomal compartment was not affected (Figure 5B, first panel) expression of Rab7da caused the formation of tight clusters of Rab7da structures in the perinuclear region that were positive for the late endosomal markers LBPA and LAMP1 (Figure 5B, second and third panel).

For this reason, GFP-Rab7da-expressing cells were used to generate a situation that represented the extreme opposite of the p50-expressing cells. Instead of redistributing endosomes to the cell periphery, endosomes were now clustered in the perinuclear region to study the effect on receptor trafficking and signal transduction.

A Tight Clustering of Endosomes Results in a Retarded Transport of EGF from EEA1 to LAMP1-positive Structures

First, we characterized the traffic of the EGFR ligand EGF in Rab7da-expressing cells by performing pulse-chase experiments. We used EEA1 as an early endosomal marker and LAMP1 as a late endosomal marker.

The EGF was transported to EEA1-positive structures in Rab7da-expressing cells (Figure 6A, first and second panel) with kinetics comparable with wild-type cells. However, the EGF was retained in EEA1-positive endosomes and not transported forward to LAMP1-positive structures. The EGF reached LAMP1-positive late endosomes after 120 min only (Figure 6B, third panel), whereas EGF colocalized with LAMP1 readily after 20 min in wild-type cells (Figure 6B, first panel). Due to this delay, the EGF was still present in Rab7da-expressing cells after 120 min, whereas in the control cells it was already degraded. Additional chase periods are shown in Supplemental Figure 3.

Figure 5. GFP-Rab7da-expressing cells show a tight clustering of the late endosomal compartment in the perinuclear region. (A) Distribution of the endosomal system in normal (left) and in GFP-Rab7da-overexpressing cells (right). (B) Confocal images of untransfected and GFP-Rab7da (green)-expressing HeLa cells are shown. Cells were subjected to indirect immunofluorescence using antibodies against EEA1 (red), LAMP1 (red), and LBPA (red). Bars, 10 μ m.

GFP-Rab7da Expression Causes a Delay in the Transport of the Activated EGFR

Next, we investigated whether the clustering of the late endosomal compartment affects the trafficking of activated EGFR, by using an antibody specific for the activated state of the EGFR (Figure 7). In the control cells, the activated EGFR had already reached the perinuclear region after a chase of 10 and 20 min, whereas in the Rab7da-expressing cells most of the EGFR was still distributed throughout the cell (Figure 7, third and fourth panel). This observation confirmed the data shown in Figure 6, A and B, where we demonstrated that EGF is retarded in EEA1-positive structures and had not yet reached the late endosomal compartment in Rab7da-expressing cells. Control cells showed only moderate activation of EGFR in the perinuclear region after a 60-min chase, which was undetectable after a 120-min chase (cells surrounded by white circles). In sharp contrast, GFP-Rab7da-expressing cells still exhibited a strong activation of EGFR after a chase of 60 and 120 min on Rab7-positive structures (Figure 6, last panel, magnification in inset). A quantitative analysis of EGFR hyperactivation is demonstrated in Figure 7B. After 120 min of chase, activated EGFR could be detected in 84% of GFP-Rab7da-expressing cells compared with only 9% in control cells. Immunoblot analysis confirmed a significant delay of EGFR degradation in Rab7da-expressing cells, especially at 20 and 60 min of stimulation (Figures 8A and 9). In the Rab7da-expressing cells, the EGFR levels were increased by 1.4-fold compared to

Figure 6. GFP-Rab7da-expressing cells show a delayed transport of EGF from the EEA1 to the LAMP1-positive structures. (A) HeLa cells expressing GFP-Rab7da were pretreated as described in legend to Figure 2, and they were labeled with an antibody against EEA1 (blue). Chase periods of 10, 20, and 60 min are displayed. Confocal images are shown. High-magnification views are shown in the rectangles. Bars, 10 μ m. (B) HeLa cells expressing GFP-Rab7da were pretreated as described in legend to Figure 2, and they were labeled with an antibody against LAMP1 (blue). Chase periods of 20, 60, and 120 min are displayed. Confocal images are shown. High-magnification views are shown in the rectangles. Bars, 10 μ m.

control cells, as already demonstrated in p50-expressing cells. However, the densitometric analysis of the EGFR/ERK degradation after the normalization to EGFR levels of control cells confirmed an altered EGFR degradation kinetic (Figure 9). The EGFR levels of Rab7da-expressing cells (grew squares) showed a dramatic delay until 60 min of stimulation then decelerated with a similar kinetic to those of control cells (white squares).

The expression of GFP-Rab7da and hence the tight clustering of the late endosomal compartment in the perinuclear region caused a delay in the trafficking of the activated EGFR from the early to the late endosomal compartment, resulting in a delay of its degradation.

A Delay in EGFR Transport Changes the Activation Pattern of MAPK Signaling Pathways

Next, we investigated how clustering of the late endosomal compartment and hence sustained activation of the EGFR is

Figure 7. GFP-Rab7da-expressing cells showed a delayed p-EGFR transport to tightly clustered perinuclear endosomes. (A) HeLa cells expressing GFP-Rab7da were serum-deprived for 14 h. Cells were preincubated with DMEM containing Alexa568-labeled EGF for 1 h at 4°C, and then they were stimulated for 10 min at 37°C (pulse), followed by a 0-, 10-, 20-, 60-, and 120-min chase in DMEM. In the absence of EGF, cells were subjected to indirect immunofluorescence analysis by using an antibody against p-EGFR, and they were analyzed by triple-channel fluorescence microscopy. Rab7da-positive cells are shown in green, EGF internalization in red, and activated EGFR in blue. Confocal images are shown. High-magnification views are shown in the rectangles. Bars, 10 μ m. (B) The graph shows the presence of the activated EGFR in HeLa cells expressing GFP-Rab7da and in control cells after a 10-min EGF pulse and 120-min chase; 84% of GFP-Rab7da-expressing cells exhibited activated EGFR after 120-min chase, whereas only 9% in control cells; 100% are equivalent to 100 cells. SD of three independent experiments is indicated.

transmitted to downstream MAPK signaling pathways. For this reason, GFP-Rab7da-expressing cells and control cells were stimulated with EGF for the indicated time periods, and immunoblots using antibodies against p-ERK and p-38

Figure 8. A delay in EGFR transport due to a tightly clustering of the late endosomal compartment changes the activation pattern of MAPK signaling pathways. (A) HeLa cells were transiently transfected with GFP-Rab7da or left untreated. After 14-h serum deprivation, cells were stimulated with 100 ng/ml EGF for the indicated times. Cell lysates were separated by SDS-PAGE and probed with the indicated antibodies. The graphs show a densitometric analysis of the p-ERK1/2 and p-p38. One representative experiment of three is shown. (B) HeLa cells expressing GFP-Rab7da were treated as described in Figure 4B. Each time point was performed twice and measured in triplets. One representative experiment of three is shown. Relative light units are normalized to protein amount.

were performed (Figure 8A). Both, GFP-Rab7da-expressing cells and control cells showed a comparable ERK1/2 activation at 10 min. In the control cells, the p-ERK signal was already decreased at 20 min, and it almost completely disappeared at 60 min. In contrast, GFP-Rab7da-expressing cells still showed a high activation of ERK1/2 at 20 min of EGF stimulation and furthermore a persistent p-ERK signal although decreased at 60 and 120 min. In the case of the p38 activation, GFP-Rab7da and control cells exhibited no differences (Figure 8A).

Surprisingly, the delayed EGFR and the sustained MAPK signaling resulted in a significant decrease (50%) of nuclear ERK signaling as revealed by an Elk-1-driven luciferase assay (Figure 8B), which was just the opposite of the result obtained with p50-expressing cells (Figure 4B).

Thus, sustained activation of the EGFR in the perinuclear region is not translated into nuclear signaling activity, which

is in strong contrast to EGFR-trapped endosomes in the periphery of the cells.

Electron Microscopy Shows EGFR Delivery to the Internal Membranes of LAMP1-positive Compartments in p50- and Rab7da-expressing Cells

We could clearly demonstrate a delay of the EGFR degradation in p50- and Rab7da-expressing cells, respectively. To clarify whether the EGFR in p50 and Rab7da-overexpressing cells is properly sorted into luminal vesicles or retained at the limiting membrane of late endosomes, we performed double and triple immunogold labeling of thawed ultrathin cryosections for electron microscopy (Figure 10, A and B, and Supplemental Figures 5 and 6). We transiently transfected HeLa cells with GFP-p50 or HA-p50 or with GFP-Rab7da, and we studied late time points (60 and 120 min) of

Figure 9. HA-p50- and GFP-Rab7da-expressing cells display an altered EGFR degradation. HeLa cells were transiently transfected with GFP-Rab7da, HA-p50, or left untreated. After 14-h serum deprivation, cells were stimulated with 100 ng/ml EGF for the indicated times. Cell lysates were separated by SDS-PAGE and probed with EGFR and ERK antibodies. The graphs show a densitometric analysis of the EGFR levels adjusted to the corresponding ERK levels and normalized to the levels of the control cells at time point 0. HA-p50 and GFP-Rab7da cells showed a 2.4-fold and 1.4-fold increase in EGFR levels, respectively, compared with control cells. SE is indicated of five (HA-p50), seven (GFP-Rab7da), and 11 (control) independent experiments.

EGFR trafficking. Transfected cells were identified by anti-GFP immunolabeling of the GFP-Rab7da construct at the perimeter membrane of late endosomes (Figure 10B and

Figure 10. Immuno-EM of HeLa cells overexpressing (A) GFP-p50 or (B) GFP-Rab7da after 120 min of EGF stimulation. (A) p50-expressing cells show colocalization of EGFR (5-nm gold, marked by arrows) and LAMP1 (10-nm gold) at the boundary of and inside late endosomes/lysosomes. Cytoplasmic labeling of p50-GFP within the same cell locates outside the frame shown here. The neighboring multivesicular body (marked by asterisk) is free of labeling upon 120 min of EGF stimulation. Bar, 100 nm. (B) Rab7da-overexpressing cells also show EGFR labeling clearly within Rab7da-positive late endosomes/lysosomes upon 120 min of EGF stimulation (EGFR, 5-nm gold, arrows; GFP, 20-nm gold). Bar, 100 nm.

Supplemental Figure 6, A and B), and, for p50, by anti-HA or anti-GFP labeling in the cytoplasm. In both p50- and Rab7da-expressing cells, certain late endosomal/lysosomal compartments were considerably enlarged compared with respective compartments in the controls and compared with MVBs and early endosomes in general (Figure 10; also see Supplemental Figures 4, A–E, and 6, A–C vs. Supplemental Figures 4F and 5, A and B). The large late endosomes and lysosomes proved LAMP1 and EGFR positive, as shown for p50-expressing cells in Figure 10A and Supplemental Figure 6B. The normally sized MVBs and early endosomes within the same cells did not show EGFR labeling at these time points (Figure 10A; LAMP1, 10-nm gold; EGFR, 5-nm gold highlighted by arrows). Concerning the ultrastructural distribution of EGFR immunogold labeling upon 60 and 120 min of stimulation no major differences between p50- and Rab7da-expressing cells were observed. EGFR labeling clearly located inside the enlarged late endosomes and lysosomes associated with vesicular or other membranous structures and at their perimeter membrane (Figure 10, A and B, and Supplemental Figure 6, A–C). In nontransfected controls, however, late endosomes/lysosomes were EGFR negative at these late time points (Supplemental Figure 5, A and B).

Clearly, there was a certain variation throughout the here studied endosomal populations with respect to the relative intensity of EGFR immunogold labeling of perimeter membrane versus internal membranes. In our case, however, the suboptimal structural preservation of the large late endosomes in p50-transfected cells did not allow further quantitative analysis of EGFR distribution at the level of endosomal subdomains (note that including glutaraldehyde in the fixative or use of other antibodies against EGFR [see Stang *et al.*, 2004] proved unsatisfactory).

DISCUSSION

We demonstrated that the distribution and function of the late endosomal compartment controls RTK trafficking and by providing, by a yet unknown mechanism, spatial information on the activation kinetics of MAPK signaling pathways. We used two opposing approaches to mislocalize late endosomes. First, we shifted the late endosomal compartment to the cell periphery by using the dynein subunit p50. Upon overexpression of GFP-p50 in HeLa cells, late endosomes (LAMP1- and LBPA-positive compartments) and lysosomes (LysoTracker-accessible structures) accumulated at the cell periphery, whereas control cells (nontransfected cells) showed a typical perinuclear distribution, which was consistent with previously published data (Echeverri *et al.*, 1996; Burkhardt *et al.*, 1997; Valetti *et al.*, 1999). In our case, we found only minor differences in the distribution of the early, EEA1-positive compartment. It is reported that upon p50 overexpression, early and recycling endosomes also redistribute away from the center of the cell but not as dramatically as late endosomes and lysosomes. Endosomes stained for EEA1 are dispersed evenly throughout the cell, which suggests a distinct behavior of early and recycling endosomes (Valetti *et al.*, 1999). These data encouraged us to use p50-overexpressing cells as an appropriate model for our further investigations.

As a complementary approach, we clustered late endosomal structures in proximity of the nucleus by expressing the dominant-active version of Rab7, EGFP-Rab7Q67L. EEA1-positive structures exhibited a normal distribution in these cells, whereas LAMP1-, LBPA-, and GFP-Rab7da-positive organelles were tightly clustered in the perinuclear region. In accordance with our data, Bucci *et al.* (2000) reported that these organelles are positive for Lamp-1 and -2 and cathep-

sin D. Furthermore, they demonstrated that these structures are acidic (LysoTracker Red), and they can be reached by fluorescent low-density-lipoprotein-cholesterol, EGF, and concavalin A (Bucci *et al.*, 2000). Additionally, we investigated the effect of the expression of the dominant-negative mutant EGFP-Rab7T22N on EGFR internalization and degradation (data not shown). This defect mutant of Rab7 is found in the cytoplasm. EEA1-positive compartments show a normal distribution, whereas Lamp-1-positive compartments are more dispersed in the cytoplasm, as already shown by (Bucci *et al.*, 2000). However, we could confirm that Rab7T22N mutants had defect lysosomes that were not as accessible to LysoTracker (Bucci *et al.*, 2000) as those of control cells; therefore, this mutant was not suitable for studying the effect of late endosomal redistribution on EGFR transport and MAPK signaling.

Interestingly, p50 overexpression did not interfere with early steps in the endocytic pathway, such as ligand binding and uptake. Furthermore, p50 expression did not affect the EGFR delivery to late endosomes, although these organelles were located in a completely different site within the cell, at the periphery. The EGFR colocalized there with late endosomal/lysosomal markers, such as LBPA and LAMP1. LBPA is known as a marker for MVBs (Kobayashi *et al.*, 1998; Matsuo *et al.*, 2004), and it is also found on late endosomes and lysosomes. Recently, White *et al.* (2006) demonstrated that EGFR is trafficking through a subpopulation of MVBs that are distinct from LBPA-positive structures and they concluded most compartments labeling for both LBPA and EGFR to be rather late endosomes/lysosomes (White *et al.*, 2006). However, in p50-expressing cells, a strong colocalization of LBPA with EGF was observed. Either the majority of these compartments were late endosomes/lysosomes, or due to the expression of p50, a strong accumulation of LBPA-positive MVBs and EGF-containing late endosomes/lysosomes occurred. Furthermore, in p50-expressing cells, the activated EGFR accumulated there, which resulted in a prolonged activation on these dearranged endosomal structures. Valetti *et al.* (1999) also show a quantitative analysis of the transferrin (Tfn) cycle, revealing that dynamiting-overexpressing cells take up and recycle Tfn as efficiently as controls (Valetti *et al.*, 1999). In contrast, Driskell *et al.* (2007) reported that dynein is essential for the efficient recycling of transferrin and early endosomal positioning. Furthermore, they showed that cells injected with dynamitin antibodies still have EGF in early endosomes at the cell periphery after 30 min. This discrepancy to our data could be due to the different strategy for blocking dynein activity, which is obvious regarding the rather weak mislocalization of the LAMP1-positive compartments (Driskell *et al.*, 2007), indicating that injected antibodies block a different and maybe yet uncharacterized function of dynein.

Accordingly, Rab7da-expressing cells also showed normal EGFR trafficking to the early endosomes. However, in those cells the EGFR showed a delayed transport from the early endosomes to the late endosomes/lysosomes that were only reached after 60 min of stimulation, which also resulted in a prolonged activation of the EGFR at 120 min of stimulation. By expressing Rab7da in oocytes, Mukhopadhyay *et al.* (1997b) showed that the expression of Rab7da stimulates transport of a fluid phase marker, Horseradish peroxidase (HRP), from early to late endosomes. In addition, they reported a stimulated HRP transport between these compartments, but they did not analyze EGF-EGFR trafficking. However, their results are not in contrast to our observations. They have focused on the relative transport from early to late endosomes within 60 min. In our control cells, most of

the EGFR has at that time already reached the late endosomes and is degraded, whereas in Rab7da-expressing cells there is still relative transport from the early to the late endosomal compartment going on. In another publication, they demonstrated that the expression of Rab7wt in oocytes already accelerated the uptake and degradation of HRP (Mukhopadhyay *et al.*, 1997a). We, however, observed that in Rab7wt-expressing cells the EGF-EGFR complex was transported in a similar way as in control cells (data not shown); however, this was strongly dependent on the expression levels. In cells expressing high levels of Rab7wt, late endosomes are highly enlarged and cluster in the perinuclear region (Bucci *et al.*, 2000), which in turn altered EGFR trafficking in our experiments (data not shown). In both approaches used in the present study, we mislocalized the late endosomal compartment, which interfered with the trafficking of the EGFR and furthermore changed its degradation kinetics. However, in p50-expressing cells the EGFR was located on mislocalized late endosomes in the cell periphery, whereas in Rab7da-expressing cells the EGFR reached the late endosomal compartment temporally delayed. In both p50- (2.4-fold) and Rab7da (1.4-fold)-expressing cells, we observed higher levels of the EGFR, which indicated reduced and delayed EGFR degradation already at steady-state levels in these cells, most likely resulting in a feed-forward regulation of the EGFR levels. Interestingly, without ligand stimulation, the basic activation of downstream signaling cascades was unchanged. Furthermore, we detected opposite degradation kinetics in the p50 and Rab7da expression. We could clearly demonstrate that p50 expression did not affect EGFR delivery to late endosomes, but it slowed down the EGFR degradation kinetic on these compartments between 60 and 120 min of stimulation. In contrast, Rab7da expression caused a delay in the EGFR transport from early to late endosomes. This interference with EGFR trafficking resulted in a decelerated degradation between 20 and 60 min, when a considerable amount of EGFR remained in EEA1-positive structures. However, once the EGFR was entering the late endosomes, after 60 min up to 120 min we observed similar or even higher degradation kinetics as in control cells. One possible reason for the slow down of the EGFR degradation in p50-overexpressing cells could be that the EGFR was restrained on the perimeter membrane of late endosomes/lysosomes. However, based on our immunoelectron microscopy analyses with three simultaneous labels, we could clearly demonstrate that the EGFR is indeed transported to the internal membranes of late endosomes/lysosomes in p50 and Rab7da-overexpressing cells after 120 min of EGF stimulation. In contrast, late endosomes/lysosomes of untransfected cells were already depleted of EGFR at these late time points. Thus, p50 overexpression did not block but only slowed down EGFR degradation until the receptor is transported to the internal membranes where then signal transmission is shut down. Because p50-expressing cells do not show a delay in EGFR transport to late endosomes but a slow down in receptor degradation, it might be that the turnover from the perimeter membrane into the internal vesicles of late endosomes/lysosomes is somehow affected. Although if members of the ESCRT complexes would be functionally defect here, the overall structure of early endosomes and of MVBs in p50-overexpressing cells would not be normal in chemically fixed and rapidly cryofixed/freeze-substituted cells. Thus, we did not observe smaller internal vesicles in MVBs or tubular structures, as it was shown earlier for cells depleted of Vps24 or of Tsg101, respectively (Bache *et al.*, 2006; Razi and Futter, 2006).

To perform their function in degradation and recycling, endosomes undergo dynamic movements along microtubules and actin filaments. We have demonstrated that the spatial organization of late endosomes not only influences RTK transport and degradation but also has an impact on the molecular interpretation of MAPK signaling. Hoepfner *et al.* (2005) also reported that the overexpression of KIF16B, a plus end motor, relocates early endosomes to the cell periphery and inhibits transport to the degradative pathway. In contrast, down-regulation of KIF16B causes clustering of early endosomes to the perinuclear region, delayed receptor recycling to the plasma membrane, and accelerated degradation. They suggested that KIF16B regulates the intracellular localization of early endosomes, and thereby the balance between receptor recycling and degradation (Hoepfner *et al.*, 2005).

Valetti *et al.* (1999) already reported that p50 overexpression does not affect uptake or recycling of α 2-M but that it slowed its transport from the early to the late endosomal compartment. Interestingly, the relocation of late endosomes to the cell cortex by dynamin overexpression slows down the EGFR degradation despite the proximity of early and late endosomes. Probably, the environment of the actin-rich cell cortex inhibits the trafficking and degradation of the EGFR. Additionally, the spatial separation of the endosomal organelles might be important for their distinct functions (Gruenberg and Maxfield, 1995; Mellman, 1996). Although showing that early and late endosomes are still separated and not fused and accumulated at the cell periphery of p50-expressing cells, it might be that the mislocalization of the late endosomes leads to an inappropriate exchange of functionally important factors causing the degradation delay we observed. Endocytic transport depends also on PI3 kinases, such as the VPS34. On early endosomes, VPS34 interacts with activated Rab5 and promotes early endosome tethering, docking, and fusion (Miaczynska and Zerial, 2002). Furthermore, a particular function of VPS34 downstream of early endosomes is documented. Vesiculation of MVBs and the efficient transport of EGF receptors into intraluminal vesicles of MVB depend on VPS34 activity (Futter *et al.*, 2001). Directed endosomal trafficking to lysosomes also requires the generation of phosphoinositides by phosphatidylinositol-specific kinases (Simonsen *et al.*, 2001). Additionally, it was reported that Vps34 and p150 are interaction partners of Rab7 on late endosomes (Stein *et al.*, 2003).

Together, this further supports the idea that the intracellular localization governs the transport activity of organelles. We have demonstrated that the compartmental architecture is not only tightly coupled with endocytic traffic but also, in turn, influences signal transduction. Thus, the endosomal distribution pattern might introduce an additional level of regulating signaling specificity, next to other auxiliary factors, such as scaffold or adaptor proteins.

The two distinct EGFR trafficking situations reported here resulted in altered kinetics of the MAP kinases p-ERK and p-p38. In p50-overexpressing cells, the reduced degradation of the EGFR on the late endosomes caused a strong persistent ERK and p38 activation. In contrast, cells expressing Rab7da showed delayed trafficking of the EGFR to late endosomes, which caused an ERK activation that was less persistent as in p50-expressing cells and that did not change the activation of p38. Hence, the distinct spatial displacements of the EGFR signaling also resulted in different activation kinetics of the MAP kinases ERK and p38. Interestingly, nuclear translocation of active nuclear factor (NF)- κ B (p65) seems to be independent of dynamin interaction (Mikenberg *et al.*, 2006), and it does not change NF- κ B output. Cells respond to stress with very high levels of p38

activation. In contrast, basal and low level activity of p38 in nonstressed cells may have different functions. Because in our experiments cells did not show p38 (or ERK activation, for that matter) activation without EGF stimulation and because p-p38 levels in p50- and Rab7da-expressing cells did not even reach control levels, we can exclude that p38 activation was due to stress response. Furthermore, p38 is proposed to phosphorylate Rab5 effectors such as EEA1 under physiological conditions, and it is additionally required for ligand-dependent receptor degradation but not for internalization (Mace *et al.*, 2005; Frey *et al.*, 2006). Therefore, the different p38 kinetics in Rab7da and p50-expressing cells was probably based on the different EGFR degradation kinetics. Rab7da exhibited a fast degradation between 60 and 120 min and no prolonged activation of p38, whereas p50-expressing cells showed a slow down in the degradation of the EGFR and a more sustained ERK activation, which probably also caused the prolonged activation of p38 and a longer time in which p38 has to operate.

Elk-1 is a target for the three classes of MAP kinases, ERK, c-Jun NH₂-terminal kinase, and p38 (Buchwalter *et al.*, 2004). We found that the altered subcellular localization of EGFR signaling caused different signaling qualities, which impacted on the activation of the physiological nuclear target Elk-1. The p50-expressing cells exhibited an increase in the Elk-1 activation, whereas Rab7da-expressing cells showed a decrease in the activation. This different output of Elk-1 activation was probably due to the different EGFR degradation kinetics and the more prolonged ERK activation in p50-versus Rab7da-expressing cells. Interestingly, Adachi *et al.* (2002) demonstrated that prolonged ERK activation induced by a PTPase, Cpd5, leads to a strong nuclear p-ERK signal and a higher activation of p-Elk-1 compared with a more transient ERK activation induced by EGF. Alternatively, it is possible that the stronger activation of Elk-1 in p50-expressing cells is related to the p-p38 kinetic in these cells.

In conclusion, we demonstrate that the spatial organization of endosomes controls distinct aspects of EGFR trafficking and degradation kinetics and that it contributes to the regulation of MAPK signaling.

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