Activation of p38 Mitogen-Activated Protein Kinase Promotes Epidermal Growth Factor Receptor Internalization

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Endocytic trafficking plays an important role in the regulation of the epidermal growth factor receptor (EGFR). To address if cellular kinases regulate EGFR internalization, we used anisomycin, a potent activator of kinase cascades in mammalian cells, especially the stress-activated mitogen-activated protein (MAP) kinase subtypes. Here, we report that activation of p38 MAP kinase by anisomycin is sufficient to induce internalization of EGFR. Anisomycin and EGF employ different mechanisms to promote EGFR endocytosis as anisomycin-induced internalization does not require tyrosine kinase activity or ubiquitination of the receptor. In addition, anisomycin treatment did not result in delivery and degradation of EGFR at lysosomes. Incubation with a specific inhibitor of p38, or depletion of endogenous p38 by small interfering RNAs, abolished anisomycin-induced internalization of EGFR while having no effect on transferrin endocytosis, indicating that the effect of p38 activation on EGFR endocytosis is specific. Interestingly, inhibition of p38 activation also abolished endocytosis of EGFR induced by UV radiation. Our results reveal a novel role for p38 in the regulation of EGFR endocytosis and suggest that stimulation of EGFR internalization by p38 might represent a general mechanism to prevent generation of proliferative or anti-apoptotic signals under stress conditions.

Key words: anisomycin, EGFR, endocytosis, p38, UV

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Monoubiquitination of EGFR at multiple sites also seems to play an important role in receptor down-regulation (8). At the plasma membrane, receptor activation promotes the recruitment of Cbl, an ubiquitin ligase that mediates the ubiquitination of EGFR (9). Cbl can also interact with the endocytic machinery, thus ensuring EGFR internalization (10). At the endosomes, ubiquitin acts as a targeting signal for degradation through interaction with the multivesicular body-sorting machinery (11,12).

However, there are still several aspects of EGFR trafficking that remain controversial. One is whether receptor ubiquitination is required for internalization or if it just plays a role in the delivery of the receptor to lysosomes. It has been shown that chimeric consisting of the extra-cellular and transmembrane domains of EGFR fused to a single cytosolic exposed ubiquitin are constitutively internalized and degraded (8,12). However, other investigators have reported that receptor ubiquitination is not sufficient for EGFR endocytosis and that the role of Cbl is to link EGFR to coated pits through the interaction of Cbl with CIN85/endophilin complex (10,13). Recently, it has been suggested that ubiquitin could determine the route of EGFR internalization so that non-ubiquitinated EGFR exclusively follows a clathrin-dependent pathway, while ubiquitinated EGFR can enter into the cell through both clathrin-coated pits and caveolae (14,15).

Another open question to be resolved is the role of kinases in RTK internalization. As mentioned previously, EGF stimulation activates different protein kinases that participate in the transmission of many proliferative and differentiative signals. These protein kinases include the extracellular signal-regulated protein kinases (ERK) and
Anisomycin-Dependent Internalization of EGFR

Results

Anisomycin induces EGFR internalization

To address if the activation of MAP kinases induced by anisomycin has any effect on EGFR internalization, we made use of a chimera in which green fluorescent protein (GFP) has been attached to the carboxyl terminus of human EGFR (EGFR-GFP). This construct allowed us to easily visualize EGFR trafficking by immunofluorescence. It has been previously described that EGFR-GFP biochemical and cellular properties do not differ from EGFR-wt (34). Figure 1A shows that at stationary state, most of EGFR-GFP localized at the plasma membrane confirming that the presence of the GFP did not alter the normal distribution of the protein. Addition of EGF caused a rapid internalization of the receptor to endosomal structures as previously described (35). Interestingly, treatment with anisomycin for short periods of time also induced endocytosis of EGFR-GFP.

In order to characterize the route followed by EGFR-GFP after anisomycin treatment, we analyzed the co-localization of the receptor with different markers. As shown in Figure 1B, we found extensive co-localization of EGFR-GFP with early endosomal markers, such as EEA1 or internalized transferrin, after incubation with the drug for 15 min. In contrast, no co-localization with the late endosome/lysosomal marker CD63 was observed. Incubation with EGF for 15 min also caused redistribution of EGFR-GFP from the plasma membrane to early endosomes (see Supplementary Material, Figure S1) indicating that both compounds promote trafficking of EGFR-GFP to the same compartments.

Next, we analyzed if anisomycin had the same effect on endogenous EGFR. Control cells or cells treated with anisomycin or EGF for 15 min were fixed, stained with an antibody that recognizes the extracellular domain of EGFR and analyzed by flow cytometry. As shown in Figure 2, treatment with either anisomycin or EGF caused a reduction of approximately 70% in the amount of endogenous EGFR present at the plasma membrane. This indicated that the effect observed by using EGFR-GFP was not a consequence of protein over-expression or mistargeting of the receptor due to the presence of GFP at the cytosolic tail, and validates the use of this chimera.

To address if anisomycin causes the internalization of all cell-surface receptors, we analyzed the effect of the drug on the distribution of Tac, a cell-surface type 1 transmembrane glycoprotein that localizes at the plasma membrane, and TGFβRII, a cell-surface receptor kinase that requires ligand for internalization. As seen in Figure S2 (Supplementary Material), incubation with anisomycin for 20 min caused a robust internalization of EGFR while having no effect on the cell-surface distribution of Tac and TGFβRII. These data show the specificity of anisomycin for EGFR trafficking.

Anisomycin promotes endocytosis of EGFR through clathrin-coated pits

Treatment with anisomycin for short periods of time revealed that there were no apparent changes in the distribution of EGFR-GFP throughout the first 5 min of incubation. However, the receptor appeared at punctate clathrin-coated pits shortly after EGFR internalization (18,19). In addition, robust MAP kinase activation requires the presence of active EGFR at endosomes (20,21) and seems to promote cell survival (22,23). Therefore, trafficking of EGFR regulates signal propagation and amplification (24,25). Conversely, it has been suggested that signaling can regulate EGFR internalization, as different proteins implicated in signal transduction have been shown to interact with the endocytosis sorting machinery (26,27).

Anisomycin is a very useful tool because it selectively activates kinase cascades in mammalian cells, especially the MAP kinases (30,31). In this study, we used anisomycin to activate MAP kinases in the absence of ligand and analyzed the effect of this activation on EGFR internalization. Interestingly, we observed that anisomycin treatment induced EGFR endocytosis and that this process was independent of tyrosine phosphorylation or ubiquitination. Moreover, preincubation of the cells with SB203580, a highly specific inhibitor of p38 (32,33), or depletion of endogenous p38 by small interfering RNAs (siRNAs) treatment, abolished the anisomycin-induced EGFR internalization suggesting that this MAP kinase plays an important role in the regulation of EGFR trafficking.

Anisomycin is an antibiotic isolated from Streptomyces griseolus that inhibits protein synthesis by blocking peptidyl transferase activity in eukaryote ribosomes (29). Anisomycin is a very useful tool because it selectively activates kinase cascades in mammalian cells, especially the MAP kinases (30,31). In this study, we used anisomycin to activate MAP kinases in the absence of ligand and analyzed the effect of this activation on EGFR internalization. Interestingly, we observed that anisomycin treatment induced EGFR endocytosis and that this process was independent of tyrosine phosphorylation or ubiquitination. Moreover, preincubation of the cells with SB203580, a highly specific inhibitor of p38 (32,33), or depletion of endogenous p38 by small interfering RNAs (siRNAs) treatment, abolished the anisomycin-induced EGFR internalization suggesting that this MAP kinase plays an important role in the regulation of EGFR trafficking.
Figure 1: Anisomycin induces internalization of epidermal growth factor receptor-green fluorescent protein (EGFR-GFP). (A) HeLa cells were transfected with a plasmid encoding EGFR-GFP. Twenty-four hours after transfection, unstimulated (control) cells or cells treated with EGF (100 ng/mL) or anisomycin (60 μM) for 15 min were fixed and analyzed by confocal microscopy. (B) Cells expressing EGFR-GFP were treated with anisomycin for 15 min, fixed and stained with the indicated antibodies. For transferrin staining, cells were incubated with rhodamine transferrin for 15 min at 37 °C. In the merge image, EGFR-GFP is in green; EEA1, transferrin and CD63 are in red and yellow indicates co-localization. Scale bar represents 10 μm.

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oligonucleotides. Immunoblot analysis revealed a 92% reduction in the levels of \( m_2 \) when compared with cells transfected with control (non-silencing) siRNA. Levels of \( m_1 \) and \( m_3 \) were monitored as control for the specificity of the antisense (Figure 3C). HeLa cells depleted of \( m_2 \) showed very little internalization of transferrin-rhodamine when compared with control cells, indicating that AP2-dependent endocytosis is impaired under our experimental conditions (data not shown). As expected, depletion of \( m_2 \) abolished anisomycin-induced internalization of endogenous EGFR indicating that the endocytosis is AP2 dependent (Figure 3D,F). Finally, we analyzed the effect of clathrin siRNA. Figure 3E,F shows that reduction in the levels of clathrin caused a clear decrease in the amount of EGFR that was internalized in response to anisomycin. In contrast, depletion of GGA3, a clathrin adaptor that regulates sorting of proteins at trans Golgi network and endosomes, or treatment with non-silencing siRNA, did not affect EGFR internalization. All together, these data indicate that anisomycin induces endocytosis of EGFR through clathrin-coated pits.

**Anisomycin-induced internalization of EGFR does not require tyrosine phosphorylation or ubiquitination**

EGF binding induces EGFR dimerization, activation, autophosphorylation of specific tyrosine residues and monoubiquitination of the cytosolic tail. It has been proposed that the recruitment of the endocytic machinery, through interactions with both phosphotyrosines and ubiquitin, plays a role in the regulation of the receptor endocytosis. In Figure 4, we used antibodies against specific phosphotyrosines to follow the activation of the EGFR-GFP after EGF or anisomycin treatment. As mentioned previously, the addition of EGF for 15 min induced a clear redistribution of EGFR-GFP to intracellular vesicles. The majority of these structures was also labeled with antibodies against phosphotyrosines 845, 1068 and 1086 indicating that EGFR-GFP is activated. In contrast, incubation with anisomycin caused receptor internalization, but no activation as no staining for phosphorylated tyrosines 845, 1068 or 1086 was observed (Figure 4A). We also generated several mutants in which different residues that are phosphorylated after receptor activation were mutated to alanines, including Y845, Y974, Y1045, Y1068, Y1086 and the double mutant S1046/S1047. Figure S3, Supplementary Material, shows that none of these mutations affected the normal distribution of the EGFR-GFP or its response to anisomycin. In addition, EGFR-GFP immunoprecipitates from HeLa cells treated with EGF or anisomycin were immunoblotted with the anti-phosphotyrosine antibody 4G10 confirming that EGF, but not anisomycin, induced tyrosine phosphorylation of the receptor (Figure 4B). Anisomycin also failed to stimulate ubiquitination of EGFR-GFP as observed by immunoprecipitation followed by immunoblotting with FK2 antibody (Figure 4C). These results indicate that neither phosphotyrosines nor ubiquitin seems to be required for anisomycin-induced EGFR internalization.

**Anisomycin induces EGFR degradation**

Next, we followed the fate of internalized EGFR after anisomycin or EGF treatment. HeLa cells were treated with cycloheximide alone, anisomycin or EGF plus cycloheximide for different periods of time. Western blotting showed that there was a marked down-regulation of EGFR after 2 h of incubation with EGF. In contrast, anisomycin caused a much slower degradation of EGFR (Figure 5A). Quantification of several independent experiments revealed that the differences in the kinetics of EGFR degradation were more evident after short incubation times. For example, EGFR levels dropped a 50% after 1 h of incubation with EGF but showed little change with...
anisomycin treatment (Figure 5B). Interestingly, treatment with proteosomal inhibitors such as MG132 almost completely abolished anisomycin-induced EGFR degradation but had little effect on EGF-mediated degradation. Conversely, lysosomal inhibitors blocked EGF but not anisomycin-mediated degradation (Figure 5C). These results suggest that anisomycin does not induce delivery of EGFR to lysosomes. Instead, the receptor could cycle between plasma membrane and endosomes until it is degraded most probably through the proteosome.
although alternative mechanisms like caspase activation cannot be ruled out (36). Consistent with this idea, we observed a good co-localization between EGFR-GFP and transferrin up to 1 h following EGFR-GFP internalization (data not shown).

Anisomycin stimulates early p38 kinase activation
It has been reported that anisomycin is a selective activator of the MAP kinases (37,38). We examined if this activation takes place under our experimental conditions and if it happens quickly enough to justify the early endocytosis observed for EGFR. Total lysates of HeLa cells stimulated with either EGF or anisomycin at different times were analyzed by SDS–PAGE and immunoblotted with antibodies against activated p44/42, p38 and JNK. Total p44/42 was monitored as loading control. As previously reported, EGF stimulation induced a rapid activation of three members of the MAP kinase family (Figure 6). Anisomycin was also able to induce phosphorylation of both p38 and JNK. In contrast, anisomycin failed to increase p44/42 activation at early time-points while inducing a very low increase in the levels of phosphorylated p44/42 after 15 min of incubation. Total levels of p38 and JNK protein were not altered by anisomycin treatment (data not shown).

Pharmacologic or genetic inhibition of p38 blocks anisomycin-induced internalization of EGFR
As previously stated, EGFR-GFP appeared in punctated structures that co-localize with clathrin at approximately 7 min after anisomycin addition. This suggests that the protein responsible for the endocytosis of EGFR-GFP is probably activated prior to EGFR-GFP redistribution. The early activation of p38 induced by anisomycin (Figure 6) makes this protein an excellent candidate to play a role in EGFR-GFP internalization. In order to test this possibility, we used a specific inhibitor for p38. Pyridinyl imidazol, SB203580, is a highly specific, cell-permeable inhibitor of
p38 (32,33). This compound has been previously used to determine a role for p38 in several biological processes including UV- and anisomycin-induced c-jun and c-fos expression (37,39). SB203580 selectively inhibits p38 while having no significant effect on other related kinases such as ERK and JNK. To block the activity of p38, cells were treated with 10 μM SB203580 for 30 min at 37°C before being exposed to anisomycin for 15 min. As shown in Figure 7A, treatment with SB203580 dramatically decreased the internalization of EGFR-GFP induced by anisomycin, suggesting that p38 activation is necessary for this process. Interestingly, treatment with AG1478, a selective inhibitor of EGFR tyrosine kinase, had no effect on EGFR-GFP endocytosis, further confirming that phosphorylation of EGFR tyrosines is not required for anisomycin-induced EGFR internalization.

Next, we determined if inhibition of p38 by SB203580 had a non-specific effect on clathrin-mediated endocytosis. Figure 7B shows that preincubation with SB203580 blocked anisomycin-induced internalization of EGFR-GFP without affecting transferrin endocytosis. This result indicated that activation of p38 does not play a general role in clathrin-mediated internalization but it is required for down-regulation of specific receptors.

The requirement of p38 MAPK activation for anisomycin-dependent EGFR internalization was further analyzed by flow cytometry. FACS analysis of the EGFR surface expression indicated that preincubation with SB203580 caused a 2.5 fold reduction in the amount of endogenous EGFR internalized after 15 min of anisomycin stimulation (Figure 7C,D). Treatment with dimethyl sulphoxide (DMSO) had no effect on EGFR endocytosis (data not shown).

To corroborate that p38 is required for anisomycin-induced internalization of EGFR, we employed genetic means to selectively deplete endogenous p38. To do so, HeLa cells were transfected either with a pool of siRNAs targeting p38α (siRNA-p38α), p38β (siRNA-p38β) or a combination of both (siRNA-p38α + β). As seen in Figure 8, expression of p38α, but not that of p38β, was abrogated in siRNA-p38α
cells, while an inhibition in the expression of p38β, but not p38α, was seen in siRNA-p38β cells. Transfection of siRNA-p38α + β caused a significant reduction in the levels of both p38α and p38β, while treatment with a control non-targeting siRNA did not affect the expression of either of the two isoforms. Interestingly, abrogation of expression of either p38α or p38β reduced the efficiency of EGFR internalization after anisomycin treatment (Figure 8B). This reduction was much more robust in siRNA-p38α + β cells, where only a 10% EGFR internalization was observed after incubation with anisomycin for 15 min. All together, these results indicate that both p38α and p38β might act synergistically in the regulation of EGFR trafficking.

**p38 activation is also required for endocytosis of EGFR induced by UV radiation**

It has been reported that UV radiation can induce internalization of EGFR independent of its phosphorylation or RTK activation. The mechanism that mediates this process has not been characterized although it has been suggested that direct absorption of UV energy might induce a conformational change of EGFR (40,41). The fact that p38 can be activated by a variety of stress signals, including UV radiation, suggested that this protein could be involved in the UV-induced internalization of EGFR. Figure 9 shows that after UV radiation (200 J/m²) an extensive EGFR-GFP internalization, comparable with that seen after EGF or anisomycin stimulation, was observed. As expected, addition of SB203580 to the cells 30 min prior to UV radiation inhibited EGFR-GFP internalization which confirmed our hypothesis that p38 plays an important role in UV-induced endocytosis of EGFR.

**Discussion**

We have shown that activation of the stress-induced p38 MAP kinase by anisomycin induces a rapid internalization
Interestingly, EGFR endocytosis was independent of RTK activity and monoubiquitination of the cytosolic tail indicating that EGFR and anisomycin employ different mechanisms to promote internalization. The lack of ubiquitination prevented the delivery of EGFR to lysosomes for degradation. This observation is in agreement with recent reports that found that ubiquitination of EGFR is required for interaction with the multivesicular bodies-sorting machinery (42,43).

p38 was originally identified as a kinase phosphorylated in response to endocytic lipopolysacharides (44,45). Later, it was found that p38 activity is also up-regulated when cells are exposed to a variety of stimuli including certain growth factors, proinflammatory cytokines (46–48) and different forms of environmental stress such as UV light (35,49), heat (32) and osmotic shock (32,45,46). To date, four members of the p38 group of MAP kinases have been characterized, including p38α (50), p38β (51), p38γ (52,53) and p38δ (51). SB203580 is a selective inhibitor for both, p38α and β, but has no effect on the activity of the γ and δ isoforms. The inhibition of the anisomycin-induced EGFR internalization observed in the presence of SB203580 strongly suggests the involvement of p38α and/or p38β in this process. The additive effect of p38α and p38β silencing on EGFR internalization after anisomycin treatment corroborate that both isoforms likely play an important role in the regulation of EGFR trafficking. It is important to note that, while inhibition of p38 activation clearly prevented EGFR entry into the cells, no effect on transferrin internalization was observed, indicating that SB203580 does not cause a general block of clathrin-mediated endocytosis.

Recently, it has been shown that exposure of cells to UV radiation also induces internalization of EGFR (40), but the precise mechanism that controls this process remains unknown. Significantly, there are several similarities between UV-induced and anisomycin-induced EGFR endocytosis. For example, in both cases, EGFR internalization is processed by clathrin-coated pits, is independent of RTK activity or autophosphorylation, does not require receptor ubiquitination and does not result in lysosomal degradation of the receptor (41,54,36). In this paper, we show that activation of p38 is involved in the UV-induced internalization of EGFR, suggesting that the regulation of EGFR endocytosis by p38 can occur under physiological conditions. It has been shown that EGFR is involved in cell proliferation, survival and tumorogenesis and that preventing EGFR down-regulation facilitates cell transformation. Therefore, external stimuli that cause p38 activation could control the accessibility of EGFR at the plasma membrane preventing that way cell proliferation and survival under stress conditions.

How does p38 promote the internalization of EGFR? There is growing evidence showing that p38 plays an important role in numerous biological processes including inflammation (55,56), development (57,58), cell cycle (61), cardiomyocyte hypertrophy (62), cell differentiation (63,64), senescence (65) and tumorogenesis (66). Recently, it has been suggested that p38 might also participate in the regulation of endocytic trafficking. Cavalli
et al. (2001) (67) have reported that p38 can phosphorylate and activate guanyl-nucleotide dissociation inhibitor (GDI), a key regulator of the Rab cycle. Rab proteins are small monomeric GTPases with molecular masses in the 20–30 kDa range (68). Multiple Rabs have been shown to participate in the formation, fusion and movement of vesicular traffic intermediaries between different membrane compartments of the cell. Rabs function as molecular switches by cycling between two interconvertible forms, a cytosolic GDP-bound (inactive) form and a membrane associated GTP-bound (active) form. In addition, Rabs also cycle between membrane-bound and cytosolic states, and this cycling is regulated by GDI (69).

Rab 5 in particular seems to play an important role in the trafficking of EGFR. Several studies have established that activated EGFR modulates the GTase activity of Rab5 by targeting either GTase-activating proteins, such as RN-Tre (70), or GT exchange factors, such as RIN1 (71). The activation of Rab5 through EGFR or the expression of constitutively active mutants of Rab5 stimulates both the internalization and degradation of activated EGFR. In contrast, dominant negative Rab5 blocks EGF-stimulated receptor-mediated and fluid-phase endocytosis (72). Therefore, p38 could promote EGFR endocytosis by stimulating the activity of GDI in extracting Rab5 from the endosomal membranes and forming cytosolic GDI-Rab5 complex. Interestingly, GDI-Rab5 has been identified as a component of the machinery controlling clathrin-coated endocytic vesicle formation (73). In addition, GDI might facilitate the delivery of Rab5 to specific regions of the plasma membrane facilitating the interaction with cytoskeleton or specific effectors. In agreement with this idea, Huang et al. (74) have shown that the activation of p38 induced by metabotropic glutamate receptor agonists accelerates loss of surface amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors by stimulating the formation of GDI-Rab5 complexes.

We cannot discard the possibility that activation of p38 could also affect EGFR recycling. Recent evidence suggests that p38 can be recruited to endosomes (28) and regulates endosomal Rab5 effectors such as EEA1 (75). p38 also seems to play a negative regulatory role in Mycobacterium tuberculosis infection by modulating the recruitment of EEA1 to phagosomes and inhibiting that way phagosome maturation (76). This involvement may reflect a more general role for p38 in the regulation of intracellular trafficking. While further studies will be required to assess the specific mechanisms used by p38 to regulate EGFR internalization, our results strengthen the idea that there is a clear intercommunication between signaling and intracellular trafficking and suggest that p38 is a key player in this process.

**Materials and Methods**

**Antibodies and reagents**
The following commercial antibodies were used: mouse monoclonal anti-ubiquitin (FK2; Affiniti, Devon, UK), mouse monoclonal anti-EEA1, mouse monoclonal anti-EEA1, mouse monoclonal anti-CHC and mouse monoclonal anti-GGA3 (BD Transduction Laboratories, San Jose, CA, USA), mouse monoclonal anti-human TGF-RII (R&D Systems Inc., Minneapolis, MN, USA), mouse monoclonal to α-adaptin (AP-6; Affinity Bioreagents, Golden, CO, USA), mouse monoclonal anti-CD63 (H5C6; BD Pharmigen, San Jose, CA, USA), mouse phosphotyrosine antibody (clone 4G10; Upstate Biotechnology, Lake Placid, NY, USA), rabbit anti-GFP (MBL International, Woburn, MA, USA), mouse monoclonal and rat monoclonal anti-EGF receptor (ab3103 and ICR10; Abcam, Cambridge, MA, USA), mouse monoclonal and rat monoclonal anti-EGF receptor (ab3103 and ICR10; Abcam, Cambridge, MA, USA), rabbit polyclonal antibodies against p38, phospho-p38, p44/42, phospho-JNK, EGF-receptor, phospho-EGF receptor (Tyr845), phospho-EGF receptor (Tyr1068) and phospho-EGF receptor (Tyr1086) were obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies to m1, m2 and m3 were the kind gift of JS. Bonifacino (NIH, Bethesda, MD, USA).

Anisomycin, EGF, SB203580, AG1478, MG132, bafilomycin A1, ubiquitin aldehyde, mammalian phosphatase inhibitor cocktail and NP40 were obtained from Sigma (St. Louis, MO, USA). Rhodamine-transferrin was supplied by Molecular Probes (Eugene, OR, USA).

**Figure 9:** SB203580 inhibits UV-mediated internalization of epidermal growth factor receptor (EGFR). Control cells or cells preincubated with SB203580 for 30 min were irradiated with UV light (200 J/m²) and chased at 37 °C for 30 min. Cells were fixed and the distribution of EGFR-green fluorescent protein was analyzed by confocal microscopy. Scale bar represents 10 μm.

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Cloning and characterization of the EGFR-GFP chimera has been previously described (34). Mutations of residues in the cytosolic tail of EGFR-GFP were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The GFP-tagged dynamin2 K44A construct was a gift from M. McNiven (Mayo Clinic, Rochester, MN, USA).

The amounts of EGFR were quantified at each time-point by using the public domain NIH Image program (1.6–2) and are represented as the percentage of remaining EGFR in comparison with unstimulated cells in the same experiment. The activation of p44/42, JNK and p38 was determined by Western blotting with antibodies specific for phosphorylated, activated forms of these kinases.

Quantitative internalization assay
HeLa cells were grown to 90% confluence. Following serum starvation for 4–5 h, the cells were harvested in Cellstripper (Mediatech, Inc., Herdon, VA), washed once with cold PBS and incubated with DMSO or SB203580 (10 μM) at 4 °C for 30 min. The cells were then incubated with EGF (100 ng/mL) or anisomycin (10 μM) for the indicated times at 37 °C, and then placed on ice to stop internalization. The cells were fixed with 2% paraformaldehyde, incubated with the FITC-conjugated anti-EGFR antibody for 45 min on ice, washed and analyzed by the FACS-Calibur using CELLQUEST software (BD Bioscience, San Jose, CA). Amounts of EGFR remaining on the cell surface were defined as the specific fluorescence value, which was calculated after subtracting background (fluorescence of non-stained cells). Percent internalization was calculated where the specific mean fluorescence value of cells incubated at 37 °C with SB203580 was treated as 100%.

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Supplementary Material
Figure S1. EGFR-GFP redistributes from the plasma membrane to early endosomes after EGF stimulation. HeLa cells were transfected with a plasmid encoding EGFR-GFP. Transfected cells were incubated with EGF (100 ng/mL) for 15 min, fixed, permeabilized, immunostained with the indicated antibodies and examined by confocal fluorescence microscopy. For transferrin staining, cells were incubated with rhodamine-transferrin for 100 ng/mL for 15 min, fixed, permeabilized, immunostained with the indicated antibodies and examined by confocal fluorescence microscopy. For transferrin staining, cells were incubated with rhodamine-transferrin for 15 min at 37 °C. EGFR-GFP is in green; EEA1, transferrin and CD63 are in red and yellow indicates co-localization. Scale bar represents 10 μm.

Figure S2. Anisomycin has no effect on the distribution of Tac and TGFβRII. (A) HeLa cells expressing EGFR-GFP or Tac were left untreated or stimulated with anisomycin for 20 min. Cells were then fixed and analyzed by confocal microscopy. Bar represents 10 μm. (B) Cell surface levels of EGFR or TGFβRII were quantified by flow cytometry in unstimulated cells or cells exposed to anisomycin for 20 min. For detection of TGFβRII, HeLa cells were transiently transfected with a plasmid encoding human TGFβRII.

Figure S3. Anisomycin-induced internalization of EGFR-GFP was not affected by mutation of specific carboxy-terminal tyrosine residues to alanines. Wild-type EGFR-GFP or EGFR-GFP carrying the indicated mutations were transiently expressed in HeLa cells. Twenty-four hours after transfection, cells were either left untreated, or incubated with anisomycin for 15 min, fixed and analyzed by confocal microscopy. Scale bar represents 10 μm.

References
1. Ulrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell 1990;61:203–212.
2. Pawson T, Gish GD, Nash P. SH2 domains, interaction modules and cellular wiring. Trends Cell Biol 2001;11:504–511.

3. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000;103:211–225.

4. Waterman H, Yarden Y. Mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. FEBS Lett 2001;490:142–152.

5. Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell 2002;110:669–672.

6. Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. Curr Opin Cell Biol 1999;11:184–189.

7. Pawson T, Schlessinger J. SH2 and SH3 domains. Curr Biol 1993;3:434–442.

8. Mosesson Y, Shigematsu K, Katz M, Zwang Y, Yarden Y. Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. J Biol Chem 2003;278:21323–21326.

9. Thien CB, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2001;2:294–307.

10. Soubeyran P, Kowaniok Z, Szymkiewicz I, Langdon WY, Dikic I. Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. Nature 2002;416:183–187.

11. Katzmann DJ, Odorizzi G, Emr SD. Receptor downregulation and multi-vesicular-body sorting. Nat Rev Mol Cell Biol 2003;3:893–905.

12. Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol 2003;5:461–466.

13. Jiang X, Sorkin A. Epidermal growth factor receptor internalization through clathrin-coated pits requires Cbl RING finger and proline-rich domains but not receptor polyubiquitylation. Traffic 2003;4:529–543.

14. Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, Di Fiore PP, Polo S. Clathrin-independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci USA 2005;102:2760–2765.

15. Chen H, De Camilli P. The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin. Proc Natl Acad Sci USA 2005;102:2766–2771.

16. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 1994;79:875–884.

17. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. Cell Res 2005;15:10–18.

18. Di Guglielmo GM, Baass PC, Ou WJ, Posner BI, Bergeron JJ. Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. J Biol Chem 1996;271:8488–8492.

19. Carter RE, Sorkin A. Endocytosis of functional epidermal growth factor receptor-green fluorescent protein chimera. J Biol Chem 1998;273:35000–35007.

20. Cohen S, Fava EA. Internalization of functional epidermal growth factor receptor: receptor kinase complexes in A-431 cells. J Biol Chem 1985;260:12351–12362.

21. He YY, Huang JL, Gentry JB, Chignell CF. Anisomycin-activated protein kinase activity in A-431 cells is blocked by a dominant negative form of c-Jun. J Biol Chem 1994;269:42457–42465.

22. Hazzalin CA, Cano E, Cuenda A, Barratt MJ, Cohen P, Mahadevan LC. p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. Curr Biol 1996;6:1028–1031.

23. Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J. Characterization of the structure and function of a new mitogen-activated protein kinase (p38b). J Biol Chem 1996;271:17920–17926.

24. Ruyter SE, Cuenda A, Vanden Berghe W, Plaisance S, Lee JC, Hagemann G, Cohen P, Fiers W. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. EMBO J 1996;15:1914–1923.

25. Rossette C, Karin M. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. Science 1996;274:1194–1197.

26. Okavod MP, Thien CB, Wiederberg J, Chantary A, Huitfeldt HS, Langdon WY. UV-radiation-induced internalization of the epidermal growth factor receptor requires distinct serine and tyrosine residues in the cytoplasmic carboxy-terminal domain. Radiat Res 2004;161:688–691.

27. Longva KE, Blystad FD, Stang E, Larsen AM, Johannessen LE, Madshus IH. Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. J Cell Biol 2002;156:843–854.

28. Groudal LM, Stang E, Sorkin A, Madshus IH. Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. Exp Cell Res 2004;300:388–395.
59. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–1331.

60. Juv P, Kuo CJ, Reynolds SE, Konz RF, Raingeaud J, Davis RJ, Biemann HP, Bienis J. Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family proteases. Mol Cell Biol 1997;17:24–35.

61. Takenaka K, Moriguchi T, Nishida E. Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. Science 1998;280:599–602.

62. Tamura K, Sudo T, Senflleben U, Dadak AM, Johnson R, Karin M. Requirement for p38 alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. Cell 2000;102:221–231.

63. Engelman JA, Lisanti MP, Scherer PE. Specific inhibitor of p38 MAP kinase reduces early endosome autoantigen 1 (EEA1) recruitment. J Biol Chem 1996;271:26981–26988.

64. Geng Y, Valbracht J, Lotz M. Selective activation of the mitogen-activated protein kinase subgroups c-Jun NH2 terminal kinase and p38 by IL-1 and TNF in human articular chondrocytes. J Clin Invest 1996;98:2425–2430.

65. Hollenbach E, Neumann M, Vieth M, Roessner A, Malfertheiner P, Kuroyanagi N, Hagiwara M, Matsumoto K, Nishida E. Purification and identification of a major activator for p38 from osmotically shocked cells: activation of mitogen-activated protein kinase 6 by osmotic shock, tumor necrosis factor-alpha, and HO. J Biol Chem 1996;271:13211–13218.

66. Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, Lee JD, Tobias PS, Ulevitch RJ. Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. J Biol Chem 1996;271:26981–26988.

67. Juo P, Kuo CJ, Reynolds SE, Konz RF, Raingeaud J, Davis RJ, Biemann HP, Bienis J. Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family proteases. Mol Cell Biol 1997;17:24–35.

68. Kuroyanagi N, Hagiwara M, Matsumoto K, Nishida E. Purification and identification of a major activator for p38 from osmotically shocked cells: activation of mitogen-activated protein kinase 6 by osmotic shock, tumor necrosis factor-alpha, and HO. J Biol Chem 1996;271:26981–26988.

69. Kuroyanagi N, Hagiwara M, Matsumoto K, Nishida E. Purification and identification of a major activator for p38 from osmotically shocked cells: activation of mitogen-activated protein kinase 6 by osmotic shock, tumor necrosis factor-alpha, and HO. J Biol Chem 1996;271:26981–26988.