Regulation of epidermal growth factor receptor traffic by the small GTPase RhoB

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Members of the Rho family of small GTPases control cell adhesion and motility through dynamic regulation of the actin cytoskeleton. Although twelve family members have been identified, only three of these – RhoA, Rac and Cdc42 – have been studied in detail. RhoA regulates the formation of focal adhesions and the bundling of actin filaments into stress fibres. It is also involved in other cell signalling pathways including the regulation of gene expression and the generation of lipid second messengers [1,2]. RhoA is very closely related to two other small GTPases about which much less is known: RhoB and RhoC (which are approximately 83% identical). Perhaps the most intriguing of these is RhoB. RhoA is largely cytosolic but translocates to the plasma membrane on activation. RhoB, however, is entirely localised to the cytosolic face of endocytic vesicles [3,4]. This suggests a potential role for RhoB in regulating endocytic traffic; however, no evidence has been presented to support this. RhoA has been shown to act at the plasma membrane to regulate the clathrin-mediated internalisation of transferrin receptor [5] and of the muscarinic acetylcholine receptor [6]. We have recently demonstrated that RhoB binds the RhoA effector, PRK1 and targets it to the endosomal compartment [7]. We show here that RhoB acts through PRK1 to regulate the kinetics of epidermal growth factor receptor traffic.

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Results and discussion

Internalised receptors undergo a variety of fates. Some, such as the transferrin receptor, are recycled to the plasma membrane whereas others, such as the epidermal growth factor (EGF) receptor, are targeted to the lysosomal compartment for degradation (reviewed in [8]). These trafficking decisions are made in an early sorting compartment, which is reached by internalised receptors within 5 minutes [9]. Final commitment of the EGF receptor to degradation in the lysosome occurs in a prelysosomal compartment called the multivesicular body (MVB). Here the receptor is sorted into internal membranes by a process that involves receptor kinase activity [10]. The MVBs then fuse with the lysosomal membrane, delivering EGF receptor for degradation [11]. As RhoB has been shown to be associated with both early endosomal vesicles and MVBs [3,4], we have used a single cell assay to examine the potential involvement of RhoB in EGF receptor traffic. Before EGF treatment, the EGF receptor is located mainly in the plasma membrane (Figure 1a). Within 5–10 minutes of stimulation the receptor is clustered and internalised into small vesicles corresponding to early endosomes (Figure 1d). The receptor reaches larger perinuclear structures by 30 minutes, with some loss of signal at 60 minutes as the receptor begins to reach the lysosomal compartment (Figures 1g,j). By 120 minutes, few EGF-receptor-positive structures remain, owing to lysosomal degradation of the signal (data not shown).

Overexpression of RhoB had no effect on the distribution of EGF receptor in cells not treated with EGF nor did it affect internalisation of the activated receptor (Figure 1a,d). RhoB had, however, a marked effect on the movement of internalised receptor to the prelysosomal compartment (Figure 1g). In cells overexpressing RhoB, the EGF receptor was present at 30 minutes in a large number of small vesicles that did not concentrate around the nucleus. In this respect, its distribution resembled that at 10 minutes in either overexpressing or untransfected cells (Figure 1m), suggesting that receptor movement from early to late endosomal compartments had been retarded. Overlay of the EGF receptor and RhoB signals showed that this retardation occurred as the receptor entered the RhoB-positive compartment (Figure 2b). By 60 minutes the EGF receptor had largely exited this compartment (Figure 2c) and there was a less pronounced difference in distribution compared with untransfected cells (Figure 1j,m). Similar results were obtained with RhoB-Q63L, an activated form of the GTPase (data not shown). RhoB-T19N, an inactive form of the GTPase that is nevertheless targeted to endosomes in HeLa cells, was without effect on EGF receptor traffic, demonstrating that RhoB activation is required for this action (data not shown).

RhoA has been shown to regulate the internalisation of the transferrin receptor at the plasma membrane [5]. Given that RhoA and RhoB are essentially identical in

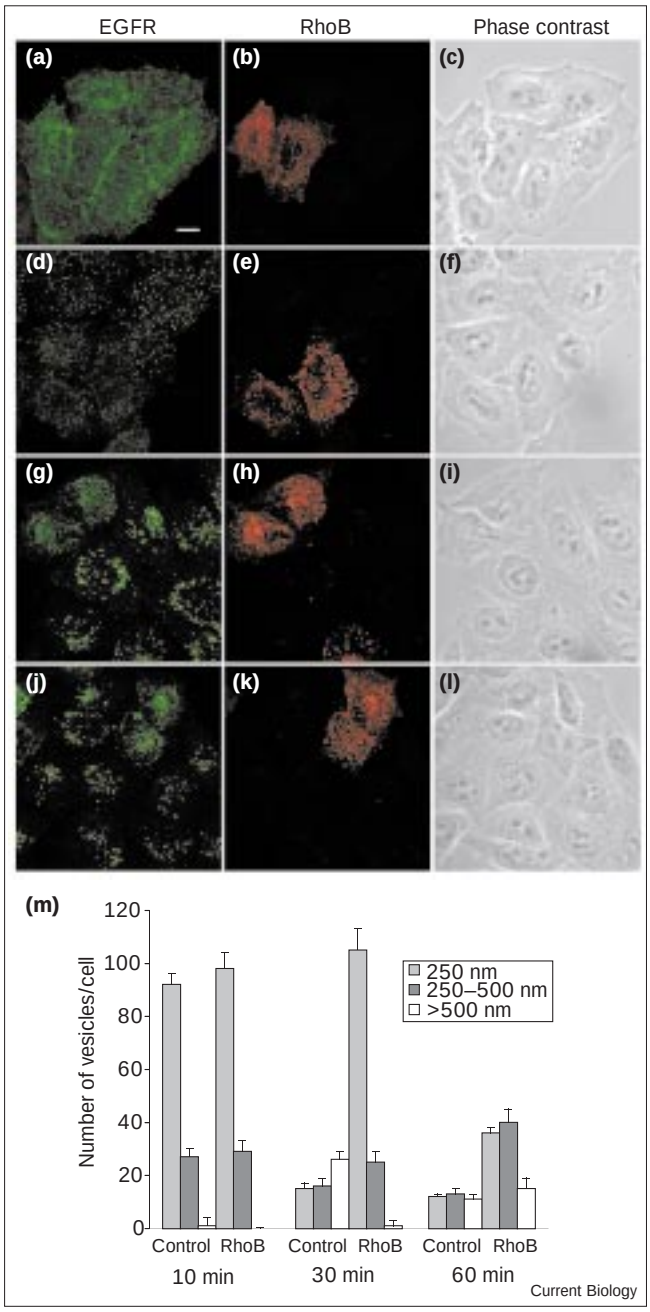


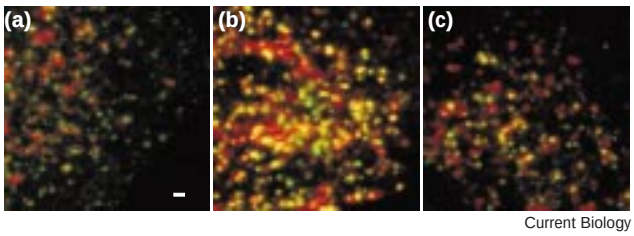
Figure 1

RhoB regulates EGF receptor (EGFR) traffic. Cells were transfected with RhoB and then treated for (a–c) 0, (d–f) 10, (g–i) 30 or (j–l) 60 min with 100 ng/ml EGF. Cells were stained for endogenous EGF receptor (green) and RhoB (red). Panels c,f,i and l show phase-contrast images of the cells. Overexpression of RhoB caused slight spreading of some cells and staining with phalloidin revealed an increase in actin stress fibres, similar to that seen with RhoA (data not shown). The scale bar represents 10 μm. (m) EGF-receptor-positive vesicles fell into three morphological classes: small 250 nm vesicles seen at early time points, intermediate sized 250 nm–500 nm vesicles seen mainly between 10 and 30 min of EGF treatment, and larger, > 500 nm, heterologous structures, seen at later time points. A series of randomised confocal images were taken of control cells and cells overexpressing RhoB at the various treatment times and imported into Adobe Photoshop. The distribution of EGF receptor between the three classes of vesicles was determined. Each data point represents 30 cells (10 cells, three experiments).

their effector-binding regions, it seemed possible that the differential localisation of the two GTPases might be the key to their action; that is, that both RhoA and RhoB might regulate endocytosis through the same effectors, with RhoA acting at the plasma membrane and RhoB in a later endosomal compartment. RhoA had no effect on the distribution of receptor 30 minutes after EGF treatment (Figure 3c). RhoA also had no effect on EGF receptor internalisation (Figure 3a), however, or indeed on any stage of EGF receptor traffic (data not shown). Similar results were seen with the constitutively activated RhoA-Q63L mutant although, as reported previously [5], this mutant totally blocked internalisation of the transferrin receptor (data not shown). Taken together, these data demonstrate that regulation of EGF receptor traffic is a RhoB-specific effect.

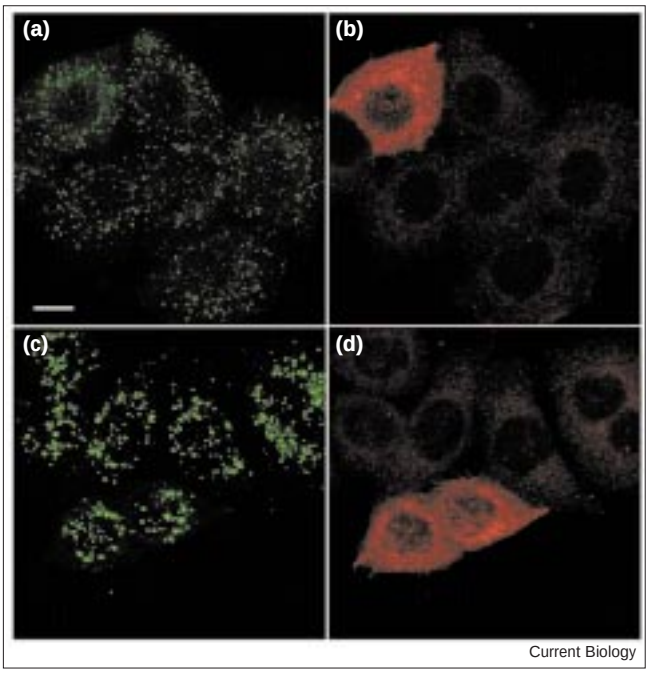
In previous work we showed that RhoB binds to the PRK1 kinase (PKN) and targets it to the endosomal compartment. Endosomal binding of PRK1 is accompanied by a shift in mobility of the kinase on SDS–PAGE, suggesting that translocation is coupled to activation [7]. Having identified an endosomal function for RhoB we investigated the potential involvement of PRK1. RhoA proteins with point mutations at residue F39 show differential binding to downstream effectors; for example, RhoA-F39A does not interact with kinases PRK1 or ROCK but still binds to the mDia2 protein. Such RhoA mutants have been used to discriminate between different downstream branches of the RhoA signalling pathways [12]. Like RhoA, RhoB-F39G was unable to bind PRK1 (Figure 4a) and overexpression of this mutant had no effect on EGF receptor traffic (Figure 4b), consistent with a potential involvement of PRK1 in this process. As some substitutions at F39 in RhoA make it unable to bind to ROCK, we investigated

Figure 2



RhoB regulates transit of EGF receptor through the RhoB-positive compartment. The figure shows overlays of sections (quadrants) of cells stained for endogenous EGF receptor (green) and RhoB (red) after EGF treatment for (a) 10, (b) 30 or (c) 60 min. Significant co-localisation (yellow) of RhoB and EGF receptor is seen after 30 min of stimulation. The scale bar represents 1 μm.

Figure 3



RhoA does not control EGF receptor traffic. Cells were transfected with RhoA and then treated for (a,b) 10 min or (c,d) 30 min with 100 ng/ml EGF. Cells were stained for endogenous EGF receptor (green) and RhoA (red). RhoA expressed at this level caused a marked increase in actin stress fibre formation as judged by phalloidin staining (data not shown); however, no effect of RhoA on EGF receptor traffic was observed. The RhoA polyclonal antibody is sensitive enough to detect endogenous protein, resulting in some staining of untransfected cells. The scale bar represents 10 μm.

whether this Rho effector was involved in the regulation of EGF receptor traffic by RhoB. Treatment of cells with the specific ROCK inhibitor Y-27632 did not block the effects of RhoB on EGF receptor traffic, and overexpression of ROCK or an activated ROCK mutant did not mimic the regulation of traffic by RhoB (data not shown).

Finally, we directly examined the role of PRK1 in RhoB signalling by coexpressing RhoB with either wild-type PRK1 or the inactive mutant PRK1-K644M. We have previously found that this approach requires careful interpretation of the results as overexpression of either wild-type or inactive PRK1 can cause inhibition of Rho signalling pathways that do not involve PRK1, such as actin stress fibre formation. This is presumably due to a titration of Rho GTPase by PRK1 away from the relevant downstream signalling protein (H.M. and P.J.P., unpublished results). This nonspecific inhibitory effect was not observed here. Coexpression of wild-type PRK1 with RhoB mildly potentiated the effects of RhoB on EGF receptor traffic (Figure 4c,i), whereas coexpression of RhoB with the kinase-dead PRK1-K644M mutant completely blocked these effects (Figures 4d,i). We conclude that RhoB signals through PRK1 to regulate the kinetics of EGF receptor traffic. This represents the first description of a function for PRK1 in mammalian cells.

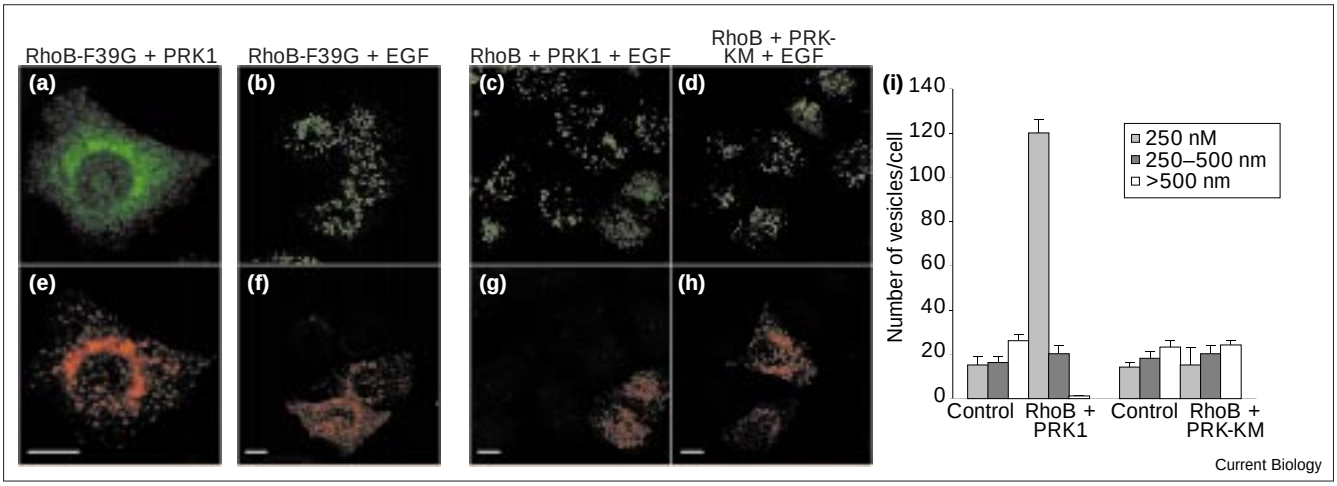
RhoA and RhoB are highly homologous at the protein level and interact with an overlapping range of downstream effectors. We have demonstrated here, however, a novel mechanism of regulating EGF receptor traffic that is mediated specifically by RhoB. Targeting of activated receptors to the lysosome represents an 'off-switch' for mitogenic signals, and the regulation of the kinetics of EGF receptor traffic by RhoB thus has potential implications for EGF signalling. Clearly, the most obvious hypothesis is that the gating of EGF receptor traffic by RhoB might prolong the active life of the receptor in the cell and therefore the duration of signalling. Other, more complicated, scenarios also need to be considered. Many mitogenic receptors show compartment-specific signalling; for example, when present at the plasma membrane, the insulin receptor signals to phosphatidylinositide 3-kinase, but it signals to the Ras-activated MAP kinase pathway only when present on internalised endosomes [13]. It is therefore possible that RhoB functions to alter the balance of signals from internalised receptors by promoting signalling from internal compartments. In this respect, it is interesting to note that the stimulation of HeLa and other cell lines with EGF causes a dramatic increase in RhoB (but not RhoA) expression that is detectable within 30 minutes and maximal after an hour [14]. This suggests a potential involvement of RhoB in mediating both acute and adaptive responses to EGF stimulation. We are currently attempting to derive single-cell assays for EGF receptor signalling to address these questions.

Materials and methods

Receptor grade EGF purified from murine submaxillary glands was from Calbiochem. The specific ROCK inhibitor Y-27632 was generously provided by Yoshitomi Pharmaceuticals Ltd (Saitama, Japan). Rabbit polyclonal antibodies to RhoA and RhoB were from Santa Cruz. The monoclonal anti-EGF receptor antibody EGFR1 and polyclonal antibody to PRK1 were as previously described [15,16]. Cy2-conjugated and Cy3-conjugated secondary antibodies were from Jackson Laboratories. Constructs encoding the ROCK-1 kinase and the activated ROCKΔ3 mutant were generously provided by Erik Sahai (Institute of Cancer Research, London).

HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). For immunofluorescence microscopy, cells were plated on acid-washed coverslips and allowed to adhere overnight. The following day, cells were transfected with various mammalian expression vector constructs (in pcDNA3; Invitrogen) using Transfast lipid (Promega). After exposure to the lipid–DNA mix for 7 h, the cells were washed into serum-free DMEM supplemented with 0.1% fatty-acid-free BSA and left for a further 14 h before experimentation. EGF treatment was carried out in the same medium supplemented with 100 ng/ml EGF. Immunofluorescent staining was carried out essentially as described in [7]. Cells were viewed using a Leica DM RBE confocal microscope equipped with a Plan APO ×63/1.36 oil immersion lens. Cy2 and Cy3 were excited using the 488 nm and 568 nm lines of a Kr–Ar laser, respectively. Series of images were taken at 0.5 μm intervals through the Z-plane of the sample and were processed to form a projected image.

Figure 4



RhoB signals through PRK1 to regulate endocytic traffic. (a,e) Cells were cotransfected with RhoB-F39G (red) and PRK1 (green): this mutant form of RhoB is unable to translocate PRK1 to the endosomal compartment. (b,f) Cells were transfected with RhoB-F39G and then treated for 30 min with EGF. Expression of the RhoB-F39G mutant (red) appeared to alter the morphology of the RhoB-positive compartment but was without effect on the traffic of internalised endogenous EGF receptor (green). Finally, cells were transfected with RhoB and either (c,g) wild-type PRK1 or (d,h) kinase-dead PRK1 (PRK-KM) and then treated with EGF for 30 min. Cells were stained for PRK1 (red) and endogenous EGF receptor (green). Overexpression of wild-type PRK1 slightly potentiated the effects of RhoB on receptor traffic (c,g), whereas overexpression of kinase-dead PRK1 blocked those effects (d,h). Overexpression of kinase-dead PRK1 alone did not have a significant effect on EGF receptor traffic (data not shown) suggesting that RhoB action is acutely regulated, rather than being a constitutively active process. The scale bar represents 10 μm. (i) The intracellular distribution of endogenous EGF receptor was determined for control cells or cells coexpressing RhoB and PRK1 or RhoB and PRK1-K644M (PRK-KM), as described in the legend to Figure 1. Each data point represents 30 cells (10 cells, three experiments).

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