The Eps8 protein coordinates EGF receptor signalling through Rac and trafficking through Rab5

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How epidermal growth factor receptor (EGFR) signalling is linked to EGF trafficking is largely unknown. Signalling and trafficking involve small GTPases of the Rho and Rab families, respectively. But it remains unknown whether the signalling relying on these two classes of GTPases is integrated, and, if it is, what molecular machinery is involved. Here we report that the protein Eps8 connects these signalling pathways. Eps8 is a substrate of the EGFR, whose activity is regulated by the EGFR. By entering in a complex with Eps8, RN-tre acts on Rab5 and inhibits internalization of the EGF receptor (EGFR). Furthermore, RN-tre diverts Eps8 from its Rac-activating function, resulting in the attenuation of Rac signalling. Thus, EGFR. Furthermore, RN-tre displays a Rab5-GAP activity on Rab5 (Fig. 1c).

Rab5 regulates trafficking in the early endocytic pathway and Rab5-GTP is required for the homotypic fusion of early endosomes in vitro. Thus, a Rab5-GAP should inhibit early endosome fusion. Indeed, endosome fusion was blocked by GST–TrH, but not by the GAP-defective TrH mutants (Fig. 1d).

Owing to its GAP activity, overexpression of RN-tre should inhibit Rab5-dependent functions in vivo, such as endocytosis of the transferrin receptor (TR)6,11. Overexpression of RN-tre, but not of the GAP-defective R150A mutant, resulted in severe impairment of transferrin internalization (Fig. 2a). Endocytosis of the TR is a constitutive process, whereas other receptors are internalized upon ligand engagement. The prototype of such receptors is the epidermal growth factor (EGF) receptor (EGFR), whose internalization might also be regulated by Rab5 (ref. 12). Indeed, a dominant-negative mutant of Rab5 (Rab5S34N) inhibited EGF internalization (Fig. 2b). Accordingly, overexpression of RN-tre also inhibited

shown to encode the catalytic core of the GTPase-activating (GAP) activity of Gyp1 and Gyp2, two yeast GTPases for Ypt/Rab GTPases. In RN-tre immunoprecipitates, we detected a Rab5-GAP activity that could be mapped to the TrH-containing region (residues 2–395) of RN-tre (see Supplementary Information). A recombinant TrH domain (glutathione S-transferase (GST)–TrH) stimulated GTP hydrolysis by Rab5, but not by Rab4, Rab11, Rac, Cdc42, Ras or Rho (Fig. 1a). Rab5 displays a higher intrinsic rate of GTPase activity than other Rab proteins (0.12 min⁻¹) (Fig. 1b). Catalytic amounts of GST–TrH increased the rate of hydrolysis to 0.46 min⁻¹ (Fig. 1b), a value in the same range of the GTPase activity measured for Rab5 on the endosome membrane in vitro. GST–TrH displayed no effect on nucleotide exchange (see Supplementary Information). There was also a direct interaction between RN-tre and GTP-bound Rab5 (see Supplementary Information).

In a general model for GAP(s) catalysis, two arginine residues are essential to stabilize the transition state of the GTP hydrolysis reaction. In TRH domains, two arginine residues (residues 106 and 150 of RN-tre) are highly conserved. In addition, an aspartate residue (residue 147 of RN-tre) is invariant. We individually mutated Arg 106, Arg 150 and Asp 147 to alanine. Consistent with the proposed mechanism, these mutants failed to display GAP activity on Rab5 (Fig. 1c).

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Figure 1 RN-tre is a Rab5-GAP whose catalytic activity is encoded by the TrH domain. a, The indicated GTPases (2 μM) were loaded with [γ-32P]GTP and incubated with GST (open bars) or purified GST–TrH (100 nM) (filled bars). b, Time-dependent kinetic of the GAP activity of GST–TrH (100 nM) (filled circles) or GST (open circles) on [γ-32P]GTP–Rab5 (2 μM). c, [γ-32P]GTP–Rab5 (2 μM) was incubated with either wild-type GST–TrH (filled circles) or GST–TrH(R106A) (filled squares), or GST–TrH(R150A) (filled triangles), or GST–TrH(D147A) (open squares) (100 nM) or with GST (open circles). d, Early endosome fusion assay. The assay was performed in the presence of HeLa cells cytosol and ATP-regenerating mix (energy) (basal = 100% fusion) or cytosol and energy plus increasing concentrations (7, 35 and 178 nM) of GST–TrH wild-type (WT) or the three GST–TrH mutants (D147A, R150A, R106A).

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EGFR endocytosis in a GAP-dependent manner (Fig. 2b). We also showed that EGFR-dependent signalling attenuates the GAP activity of RN-tre. After exposure of intact cells to EGF, we recovered significantly decreased levels of Rab5-GAP activity in RN-tre immunoprecipitates, with respect to unstimulated cells (Fig. 2c). RN-tre is phosphorylated on serine after EGF stimulation within a time-frame similar to that of the downregulation of its GAP activity (data not shown); it will be therefore of interest to determine...
whether this post-translational modification affects the enzymatic activity of RN-tre. Whatever the case, the sum of our results establishes RN-tre as a GAP that modulates the activity of Rab5 both in vitro and in vivo, and whose activity is controlled by EGFR.

RN-tre binds to the src homology (SH)-3 of Eps8 (ref. 3), an activator of Rac-specific guanine nucleotide exchange factor (GEF)1. We therefore tested whether Rab5 and Rac signalling might be co-regulated. In Eps8 null fibroblasts (Eps8−/−), over-expression of RN-tre failed to inhibit EGFR internalization, while still inhibiting TIR endocytosis (Fig. 3). In Fig. 3, we further showed (1) that in HeLa cells a mutant RN-tre (RN-tre-PXXGS), which cannot bind to Eps8 (Supplementary Information), failed to inhibit EGFR endocytosis; (2) that transfection of Eps8 in Eps8−/− fibroblasts restored the ability of RN-tre, but not of RN-tre-PXXGS, to inhibit EGFR internalization; (3) that transfection of Eps8−/− fibroblasts with a mutant Eps8, carrying a 5-amino-acid deletion in its SH3 domain (Eps8ΔSH3; ref. 13) and no longer able to bind to RN-tre (see Supplementary Information), failed to restore the effect of RN-tre on EGFR internalization; (4) that TIR internalization was still inhibited, by RN-tre or RN-tre-PXXGS, in all of the above conditions. Thus, the interaction between RN-tre and Eps8 is necessary for the regulation of ligand-dependent but not of constitutive endocytosis.

Eps8 activates Rac by stimulating the Rac-GEF activity of Sos-1 (ref. 2). In vivo, a tri-complex exists in which a scaffolding protein, E3b1, brings together Eps8 and Sos-1. All three components are needed for the complex to function as a Rac-GEF1. Eps8 interacts with both E3b1 and RN-tre through its SH3 (ref. 14). Thus, Eps8–E3b1 and Eps8–RN-tre complexes should be mutually exclusive.

Indeed, the overexpression of RN-tre decreased the co-immunoprecipitation between Eps8 and E3b1 (Fig. 4a), and resulted in the attenuation of Rac-specific signalling. Using activation of c-Jun aminoterminal kinase (JNK) as a readout, we showed that over-expression of RN-tre in Cos-7 cells resulted in a threefold decrease in EGF-induced JNK activity, but left mitogen-activated protein kinase (MAPK) activity unperturbed (Fig. 4b). Expression of the RN-tre(R150A) (R150A) mutant also inhibited JNK (Fig. 4b), suggesting that the physical interaction of RN-tre with Eps8, rather than the GAP activity, was responsible for the effect.

We validated this hypothesis in Eps8−/− fibroblasts, in which signalling from Ras to Rac, but not from Ras to Raf, is impaired2. Accordingly, a threefold reduction of the EGFR-dependent JNK activity, but not MAPK, was observed in Eps8−/− fibroblasts, as compared with wild-type cells (Fig. 4c). The persistence of some EGF-dependent JNK induction in Eps8−/− cells might reflect the existence of Rac-independent pathways that also contribute to the activation of this enzyme by growth factors15. As expected, re-expression of Eps8 in Eps8−/− cells resulted in the restoration of full EGF-dependent JNK activity (Fig. 4d). Overexpression of RN-tre in Eps8−/− fibroblasts did not modify the residual levels of EGF-induced JNK activity (Fig. 4d). However, concomitant expression of Eps8 and RN-tre in Eps8−/− cells abrogated the Eps8-induced effect on JNK activity, which was indistinguishable from that of untransfected Eps8−/− cells (Fig. 4d).

We have uncovered an unexpected link between the machinery of receptor trafficking and signalling, in that two alternative complexes, Eps8–E3b1 and Eps8–RN-tre control Rac and Rab5 signalling, respectively. Eps8, which is partly localized at the plasma

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**Figure 4** Binding of RN-tre to Eps8 results in the attenuation of Rac-mediated signalling. a, Cos-7 cells were transfected (TF) with RN-tre, Eps8 and E3b1, as indicated. Cell lysates (1 mg) were immunoprecipitated (IP) with anti-eps8 or irrelevant (ctr) sera and blotted (WB) with the indicated sera. b, Cos-7 cells were transfected with HA–JNK or HA–MAPK alone (ctr), or together with RN-tre or the RN-tre(R150A) mutant (see also Supplementary Information). JNK activity was measured by an immunocomplex kinase assay on anti-HA immunoprecipitates, using c-Jun(79) as a substrate. MAPK activity was measured by immunoblotting anti-HA immunoprecipitates with anti-phosphoMAPK (New England Biolabs). Aliquots of the reactions were also immunoblotted with anti-HA to show equal loading. c, Eps8−/− and Eps8+/+ clones (2 clones each, indicated on top) were analysed for JNK and MAPK activities by immunocomplex kinase assays on anti-JNK-1 and anti-MAPK immunoprecipitates, using c-Jun(79) and MBP as substrates. Aliquots of the reactions were also immunoblotted with specific anti-JNK and anti-ERK1 antibodies to show equal loading. d, Eps8−/− cells were transfected with the plasmids indicated on the top, together with HA–JNK and tested for JNK activity as in b (see also Supplementary Information). The levels of JNK are also shown. Results are typical and representative of three independent experiments.
membrane and physically interacts with the EGFR, is likely to recruit and coordinate the activity of the two types of signalling complexes. We have established that RN-tre is a Rab5 GAP. By acting on Rab5, RN-tre regulates both the constitutive and the regulated (for example, EGF-dependent) receptor internalization. The latter activity strictly depends on the interaction of RN-tre with Eps8. Recruitment of RN-tre would decrease Rab5 activity, thereby inhibiting receptor internalization and, consequently, prolonging receptor signalling at the plasma membrane. Alternatively, recruitment of E3b1 (and Sos1) couples the receptor with downstream Rac activation. Possibly, both Eps8–RN-tre and Eps8–E3b1 complexes are formed simultaneously and/or temporally uncoupled, which might happen under rate-limiting concentrations of Eps8 in a restricted signalling microenvironment, such as caveolae. In this last case, competition between RN-tre and E3b1 for Eps8 might result in more complex outcomes, dependent on the stoichiometry and temporal hierarchy of the two interactions. Whatever the case, our results establish a new modality of integration of signals emanating from Rho-like and Rab-like GTPases: an unexpected connection that warrants further investigation.

Methods

Expression vectors and antibodies

We engineered haemagglutinin A (HA)-, green fluorescent protein (GFP)- or GST-tagged full-length RN-tre, truncation and point mutants obtaining appropriate fragments by recombinant PCR, or by endonuclease digestion, followed by subcloning in the pcDNAHAI (ref. 19), pEGFP-C1 (Clontech), or pGEX-4T2 (Pharmacia) vectors.

We engineered the RN-tre retrovirus by cloning the untagged full-length RN-tre in the pBMN retroviral vector, under the control of the CMV promoter. This vector also drives the transcription of the GFP gene from a LTR promoter. After rescue of infectious retroviruses, infection of HeLa cells was performed under conditions yielding more than 80% of infected cells, as assessed by expression of GFP.

We obtained recombinant small GTPases of the Rab family by subcloning the appropriate recombinant GTPase fragments into pGEX vectors. All constructs were verified by DNA sequencing. Further details are available upon request. Antibodies used were an anti-HA monoclonal antibody (12CA5, BABCO) and affinity purified anti-RN-tre3, anti-Eps8 and anti-E3B1 for Eps8 might result in more complex outcomes, dependent on the stoichiometry and temporal hierarchy of the two interactions. Whatever the case, our results establish a new modality of integration of signals emanating from Rho-like and Rab-like GTPases: an unexpected connection that warrants further investigation.

Biochemical and functional assays

For GAP assays on immunoprecipitates, 6 mg of total cellular lysates were immunoprecipitated with anti-HA and subsequently incubated for 5 min at 20°C in the presence of [γ-32P]GTP-gamma-sulphone (0.15 μM). Incubation mixtures were subjected to filter-binding assays as described.

We carried out GAP assays with GST–TrH as described, and anti-HA monoclonal antibody (12CA5, BABCO) and affinity puriﬁed anti-RN-tre, anti-Eps8 and anti-E3B1 for Eps8 might result in more complex outcomes, dependent on the stoichiometry and temporal hierarchy of the two interactions. Whatever the case, our results establish a new modality of integration of signals emanating from Rho-like and Rab-like GTPases: an unexpected connection that warrants further investigation.

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Deacetylation of p53 modulates its effect on cell growth and apoptosis

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The p53 tumour suppressor is a transcriptional factor whose activity is modulated by protein stability and post-translational modifications including acetylation. The mechanism by which acetylated p53 is maintained in vivo remains unclear. Here we show that the deacetylation of p53 is mediated by an histone deacetylase-1 (HDAC1)-containing complex. We have also purified a p53 target protein in the deacetylase complex (designated PID; but identical to metastasis-associated protein 2 (MTA2)), which has been identiﬁed as a component of the NuRD complex. PID speciﬁcally interacts with p53 both in vitro and in vivo, and its expression reduces signiﬁcantly the steady-state levels of acetylated p53. PID expression strongly represses p53-dependent transcriptional activation, and, notably, it modulates p53-mediated cell growth arrest and apoptosis. These results show that deacetylation and functional interactions by the PID/MTA2-associated NuRD complex may represent an important pathway to regulate p53 function.

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