RESEARCH COMMUNICATION

Small interfering RNAs as a tool to assign Rho GTPase exchange-factor function *in vivo*

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Rho GTPases control a complex network of intracellular signalling pathways. Whereas progress has been made in identifying downstream signalling partners for these proteins, the characterization of Rho upstream regulatory guanine-nucleotide exchange factors (GEFs) has been hampered by a lack of suitable research tools. Here we use small interfering RNAs (siRNAs) to examine the cellular regulation of the RhoB GTPase, and show that

RhoB is activated downstream of the epidermal-growth-factor receptor through the Vav2 exchange factor. These studies demonstrate that siRNAs are an ideal research tool for the assignment of Rho GEF function *in vivo*.

Key words: endocytosis, epidermal growth factor, small interfering RNA (siRNA).

INTRODUCTION

The Rho family of small GTPases play a pivotal role in the control of cell movement, cell morphology and cell adhesion through dynamic regulation of the actin cytoskeleton [1,2]. These signalling proteins also mediate a diverse set of other cellular functions, including control of cell-cycle progression, gene transcription and intracellular membrane traffic [3–5].

Rho GTPases act as molecular switches in signalling pathways, converting between an inactive closed conformation and an active open one. Binding of GTP triggers conversion into the active conformation, and this then allows the protein to interact specifically with its downstream effector(s). The signal is terminated through hydrolysis of GTP, returning the Rho GTPase to a closed GDP-bound state. The intrinsic rates of exchange and hydrolysis on Rho GTPases are low, and two classes of accessory proteins act to control this cycle: guanine-nucleotide exchange factors (GEFs) activate Rho GTPases by catalysing the exchange of bound GDP for GTP; GTPase activating proteins ('GAPs') then turn signalling off by stimulating the hydrolysis of GTP on the Rho GTPase, returning the Rho to its inactive conformation. Significant progress has been made in identifying downstream signalling partners for Rho GTPases, which have been isolated through techniques exploiting the tight and specific binding of activated Rho GTPases for their targets [2,6]. However, our knowledge of the upstream components of Rho GTPase signalling pathways is poor by comparison. Progress has been hampered by the difficulties of establishing the *in vivo* specificities of Rho GEFs and their linkage to particular signalling pathways. Clear assignment of Rho GEF function has generally only been possible with studies of human disease states that involve deletion of a specific Rho GEF, such as Aarskog–Scott syndrome (facio-genital dysplasia) [7] or from targeted deletion of Rho GEFs in transgenic mouse lines [8–10]. A further complication is the size of the Rho GEF family. There are at least 23 human Rho GTPases [11], but these are regulated by more than 70 human Rho GEFs [9,10]. Although understanding Rho GEF function is clearly an important research goal, the available methodologies are not well suited to dissecting such a complex network of signalling pathways.

Tuschl and co-workers have recently shown that small interfering RNAs (siRNAs) can be used to specifically down-regulate the expression of target genes in cultured mammalian cells [12]. Here we use siRNAs to dissect signalling pathways upstream of the RhoB GTPase and show that this approach is ideally suited to the assignment of Rho GEF function *in vivo*.

MATERIALS AND METHODS

Materials

Monoclonal antibodies to RhoB, RhoA and glutathione S-transferase (GST) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Fluorescent-dye-Cy3-conjugated donkey anti-rabbit IgG was from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.). The rabbit polyclonal antibody to exchange factor Vav2 was generously provided by Dr Xosé Bustelo (Centro de Investigacion del Cancer and Instituto de Biologia Molecular y Celular del Cancer, University of Salamanca-CSIC, Salamanca, Spain). Human recombinant epidermal growth factor (EGF) was from Calbiochem (San Diego, CA, U.S.A.). Lysophosphatic acid (LPA) was from Sigma (St Louis, MO, U.S.A.).

Preparation of GST-fusion proteins

Human Rho GTPases were expressed as recombinant GST-fusion proteins in *Escherichia coli*. pGEX-RhoA and pGEX-RhoC expression plasmids were generously provided by Dr Anne Ridley (Department of Biochemistry and Molecular Biology, University College, London, U.K.). The cDNA encoding human RhoB was subcloned into pGEX-4T3 (Amersham Pharmacia Biotech) using standard procedures. GST-fusion proteins were expressed in *E. coli* strain BL21(DE3)pLysS and purified by affinity chromatography on GSH–Sephacrose (Amersham Pharmacia Biotech) using standard procedures [13]. The Rho-binding domain of rho-kinase (TRBD) was expressed as a recombinant fusion with GST in *E. coli* as previously described [14]. Briefly, 4 litres of cells were grown to an attenuation (D_{600}) of 0.8 and protein expression was induced over 2 h by addition

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth-factor; GEF, guanine-nucleotide exchange factor; GST, glutathione S-transferase; LPA, lysophosphatidic acid; siRNA, small interfering RNA.

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Figure 1 Activation of RhoB through EGF stimulation

(A) The specificities of monoclonal antibodies to RhoA and RhoB were tested by Western blotting against purified recombinant RhoA, RhoB and RhoC. The relative loading of each GST-fusion protein (approx. 1 μ g) was assayed by blotting with an antibody to the GST tag. (B) Activation of endogenous RhoB in response to stimulation of cells with 100 ng/ml EGF was assayed using a modified pull-down assay as described in the Materials and methods section. The lower panel shows aliquots of the extracts before assay as a control for equivalent input into the assay. The figure is representative of four independent experiments where essentially identical results were obtained. (C) Densitometric analysis of the time course of RhoB activation in response to EGF. Activation was normalized in each case to total RhoB in the sample, and each bar represents the mean $\pm$ SEM ($n = 3$).

of 0.5 mM isopropyl β -D-thiogalactopyranoside. The cells were harvested by centrifugation and resuspended in 40 ml of ice-cold lysis buffer [50 mM Tris (pH 7.5)/0.5% (v/v) Triton X-100/150 mM NaCl/5 mM MgCl₂/1 M dithiothreitol/0.1 mM PMSF], and the cells were then broken by sonication. Insoluble material was removed by centrifugation and GST-TRBD protein was purified through binding to GSH-Sepharose beads. Purified GST-TRBD protein was stored on beads in aliquots at -70°C .

RhoB-activation assays

Determination of endogenous RhoB activation was carried out through modification of a procedure previously formulated for RhoA [14]. HeLa cells (approx. 2×10^6 cells) were seeded on to 10 cm² tissue-culture dishes and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. On reaching approx. 80% confluence, the cells were transferred to serum free DMEM supplemented with 0.1% fatty-acid-free BSA and incubated in this medium for 18 h prior

to experimentation. Cells were stimulated in the same medium with either 100 ng/ml EGF or 10 μ M LPA for various times and then harvested by scraping into 800 μ l of ice-cold extraction buffer [50 mM Tris (pH 7.2)/1% (v/v) Triton X-100/0.1% (w/v) SDS/500 mM NaCl/10 mM MgCl₂/aprotinin (10 μ g/ml)/leupeptin (10 μ g/ml)/0.1 mM PMSF]. Cell lysates were cleared by centrifugation at 15000 g for 10 min at 4°C . An aliquot (20 μ l) from each lysate was removed as a control for equivalent input into the assay and the remaining lysate was then combined with 15 μ l (packed volume) of GST-TRBD beads (equivalent to approx. 30 μ g of immobilized GST-TRBD protein) and rotated for 45 min at 4°C . Beads were washed four times with 600 μ l of ice-cold wash buffer [50 mM Tris (pH 7.2)/1% (v/v) Triton X-100/150 mM NaCl/10 mM MgCl₂/aprotinin (10 μ g/ml)/leupeptin (10 μ g/ml)/0.1 mM PMSF]. Bound protein was eluted from the beads with 50 μ l of SDS/PAGE sample buffer at 95°C . Proteins were resolved by SDS/PAGE and RhoB was detected by Western blotting with specific antisera. In some experiments parallel samples were processed to detect activation of endogenous RhoA, using RhoA-specific antisera.

EGF-receptor traffic

Cells for immunofluorescence were plated on to acid-washed glass coverslips and starved overnight in serum-free DMEM, containing 0.1% fatty-acid-free BSA, prior to experimentation. The cells were then given a 10 min pulse of biotinylated EGF, complexed with Alexa 488-streptavidin (Molecular Probes, Eugene, OR, U.S.A.; Alexa 488 is a fluorescent dye) in the same medium, followed by various chase periods in the absence of EGF. The fluorescent EGF complex was used at a final concentration of 1 μ g/ml, equivalent to 100 ng/ml unmodified EGF. Cells were fixed in fresh 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min and then incubated in 0.1% NaBH₄ in PBS for 10 min. After fixation, the coverslips were incubated with primary antibodies in PBS containing 1% BSA for 1 h, and then with secondary antibodies in PBS for 45 min. The coverslips were inverted on to Mowiol 4-88 (an anti-fade agent from Calbiochem) containing 0.6% 1,4-diazabicyclo[2.2.2]octane as an anti-photobleaching agent. Cells were viewed using a Leica DM RBE confocal microscope under a Plan Apo $\times 63/1.32$ oil-immersion objective. Alexa 488 and Cy3 were excited using the 488 nm and 568 nm lines of a krypton/argon laser respectively. A series of images was taken at 0.5 μ m intervals through the Z-plane of the cell and processed to form a projected image.

siRNA treatment

An siRNA against human Vav2 was designed using criteria set out by Tuschl and co-workers [12]. The coding sequence of Vav2 was scanned to identify 21 nt sequences commencing with a diadenine and comprising approx. 50% GC content. Candidate sequences were compared with the cDNA sequences of Vav1 and Vav3 and also searched against the non-redundant human DNA database using the BLAST algorithm [15]. A Vav2-specific sequence 5'-GGACAUCACUCCGGCCGdTdT-3' and its corresponding complement 5'-CGGCCGGAAGUUGAUGUCCdTdT-3' were synthesized as synthetic oligonucleotides (Dharmacon Research Inc., Lafayette, CO, U.S.A.) and annealed to form a short double-stranded RNA with a 3'-dithymidine overhang. Control siRNAs were also synthesized by Dharmacon and were as follows: control 1, 5'-CGUACGCGGAUACUUCGAdTdT-3' is directed against a non-mammalian gene (luciferase); control 2, 5'-GAACCACUUGAUGAGGACGdTdT-3' is a random oligonucleotide of similar base composition

to the Vav2 siRNA. SiRNAs were introduced into HeLa cells by lipid-mediated transfection using OLIGOFECTAMINE™ (Invitrogen, Paisley, Renfrewshire, Scotland, U.K.). For each 10 cm² tissue-culture dish, lipid–RNA complexes were formed using 1.2 nmol of RNA and 60 µl of OLIGOFECTAMINE™ lipid in OptiMem serum-free medium (Life Sciences, now Invitrogen). The lipid complex was added dropwise to the HeLa-cell cultures and incubated with the cells for 48 h. The lipid/siRNA mix was removed on transfer of cells into serum-free media, 18 h prior to experimentation.

RESULTS

Small GTPases of the Rab and Arf families have well-established roles in the regulation of intracellular membrane traffic [16,17]. However, there is emerging evidence for specific functions for Rho GTPase family members in regulating these processes [5,18]. In our previous studies we have shown that the endosomal RhoB GTPase acts to control the intracellular traffic of the EGF receptor [19]. Whereas Rab GTPases are assumed to have a processive function in trafficking pathways, Rho GTPases are generally acutely regulated signalling proteins that are activated in response to extracellular stimuli. This raises the possibility of adaptive/reactive regulation of endocytic traffic. In order to understand the physiological relevance of RhoB regulation of EGF receptor traffic, we attempted to identify the upstream signals controlling this pathway.

In order to measure activation of endogenous RhoB in cells, we adapted the RhoA-activation assay of Ren and Schwartz [14]. This assay makes use of the ability of the RhoA-binding domain of rhotekin to bind with high affinity to activated RhoA [20]. In the assay, immobilized recombinant RhoA-binding domain is used as an affinity probe to isolate active RhoA from cell lysates. The bound protein is analysed by Western blotting with anti-RhoA antisera, and the signal then corresponds to the activated fraction of RhoA from the cell lysate. RhoB is highly homologous with RhoA (87% identical) and also binds to rhotekin in an activation-dependent manner [20]. We used antibodies specific to RhoB to adapt the RhoA assay to the measurement of RhoB (Figure 1A). Of the various ligands tested, we were surprised to

find that EGF stimulation itself caused potent activation of RhoB in cells (Figures 1B and 1C). LPA (see Figure 3B) and thrombin (results not shown) also caused RhoB activation.

Activation of RhoB in response to EGF was biphasic, with an early peak in activity at 3 min, followed by a second peak approx. 60 min after stimulation (Figures 1B and 1C). Studies of the cellular localization of epitope-tagged over-expressed RhoB by immunoelectron microscopy have shown evidence for two cellular pools of RhoB: a plasma-membrane fraction and a fraction localized to multivesicular late endosomes [21]. We observed a similar distribution of the endogenous protein by immunofluorescence microscopy (results not shown). The EGF receptor takes a well-described intracellular route on activation, appearing first in early endosomes; it is then sorted into late multivesicular endosomes before being delivered to the lysosomal compartment, where it is degraded [22]. The timings of the early and late peaks of RhoB activation correspond to the activation/internalization of the EGF receptor at the plasma membrane and its subsequent appearance in RhoB+ late endosomes respectively ([22]; Figure 2). These data suggest that the activated EGF receptor mediates activation of the two cellular pools of RhoB in a sequential manner. Two pieces of correlative evidence support this hypothesis. (i) RhoA, which is localized solely to the plasma membrane and cytosol [23], showed only the rapid peak of activation in response to EGF stimulation (Figure 3A). (ii) Activation of RhoB through another stimulus (LPA) showed a rapid activation of RhoB, but did not generate the second peak of activation seen with EGF (Figure 3B). LPA causes potent activation of RhoA through Edg2, Edg4 and Edg7 LPA receptors [24]. Although little work has been done on the intracellular sorting of this family of receptors, Edg1 has been shown to recycle to the plasma membrane after activation, rather than following the degradative late-endosomal route taken by the EGF receptor [25]. If this holds for the other Edg receptor isoforms, then the activated LPA receptors would by-pass the RhoB+ late-endosomal compartment on their intracellular sorting route. Taken together, these data suggest that the two cellular pools of RhoB behave autonomously, and that activation of the late-endosomal pool of RhoB requires the physical presence of the activated EGF receptor in this compartment.

Figure 2 Transit of internalized EGF through the RhoB+ compartment

Cells were serum-starved overnight and then stimulated with a 10 min pulse of Alexa 488-conjugated EGF (green). The cells were either washed and fixed immediately (**A**), or first chased into fresh medium for 20 min (**B**) or 50 min (**C**). Fixed cells were stained for endogenous RhoB (red). The bar represents 10 µm.

Figure 3 Transitory activation of RhoA in response to EGF and RhoB in response to LPA

(A) Activation of endogenous RhoA in response to stimulation of cells with 100 ng/ml EGF. (B) Activation of endogenous RhoB in response to stimulation of cells with 10 μ M LPA. In each panel, assays were performed using the pull-down assay described in the Materials and methods section with the appropriate Rho antisera. The lower panels show aliquots of the extracts before assay as a control for equivalent input into the assay. Each panel is representative of three independent experiments where essentially identical results were obtained.

We next sought to identify the Rho GEF linking EGF receptor activation to RhoB. Although over 70 human Rho GEFs have been identified to date, little functional characterization has been achieved. However, the three Vav isoforms (Vav1–Vav3) are known to be phosphorylated in response to EGF stimulation and are recruited to the activated receptor [26,27], making them potential candidate GEFs for the regulation of RhoB. Vav1 is

Figure 4 Identification of Vav2 as a RhoB GEF using siRNA

(A) HeLa cells were transfected with control siRNAs or a specific siRNA directed against Vav2, as described in the Materials and methods section. Suppression of Vav2 was assayed by Western blotting of total cell lysates with a specific Vav2 antiserum. (B) HeLa cells were transfected with either control siRNA [control 2, top panels] or a Vav2-specific siRNA, and then stimulated with LPA or with EGF as previously described. Activation of endogenous RhoB was assayed using the pull-down assay described in the Materials and methods section. The Figure is representative of four independent experiments where essentially identical results were obtained.

Figure 5 Activation of RhoA through EGF stimulation is not mediated by Vav2

HeLa cells were transfected with either control siRNA [Control 2, top panels] or a Vav2-specific siRNA, and then stimulated with EGF as previously described. Activation of endogenous RhoA was assayed using the modified pull-down assay described in the Materials and methods section. The Figure is representative of five independent experiments where essentially identical results were obtained.

the best studied of the three isoforms, and phosphorylation of a specific tyrosine residue (Tyr¹⁷⁴) in response to EGF-receptor activation has been shown to activate its Rho GEF function by relieving an autoinhibitory contact with the catalytic site [28]. However, the expression of Vav1 is restricted to cells of haematopoietic origin [29], and we were unable to detect expression of Vav1 in HeLa cells (results not shown). Vav3 has a broader tissue distribution, but is expressed mainly during mitosis, where it functions in cell-cycle regulation [30]. Vav2 is widely and constitutively expressed [30,31] and so appeared to be the best candidate of the three for further investigation.

In order to block Vav2 function *in vivo*, we designed a Vav2-specific siRNA and introduced this reagent into cells by lipid-mediated transfection. The Vav2 siRNA suppressed expression of Vav2 to approx. 10 % of control levels within 48 h of treatment (Figure 4A). We next examined the effects of the Vav2 siRNA on RhoB activation in cells.

Down-regulating Vav2 expression had no effect on the activation of RhoB by LPA (Figure 4B). This was to be expected, as LPA receptors are coupled to heterotrimeric G-proteins [24], and activation of Rho GTPases in response to LPA is thought to involve GEFs, such as p115, that contain a conserved regulator-of-G-protein signalling ('RGS') motif [10]. However, treatment with the Vav2 siRNA revealed a role for Vav2 in EGF-receptor signalling to RhoB that was surprising; down-regulating Vav2 expression had no effect on the first peak of RhoB activation, but completely blocked the second (Figure 4B). This further suggests that the two pools of cellular RhoB are autonomous signalling compartments, and demonstrates that Vav2 is the GEF linking EGF-receptor stimulation to activation of the second RhoB pool.

Finally, we used the Vav2 siRNA to examine the role for this GEF in EGF-mediated activation of RhoA. Vav2 has been shown to be capable of acting as a RhoA GEF *in vitro* [32], and overexpression of activated mutants of Vav2 causes RhoA activation *in vivo* [33]. Taken together, this has led to the assumption that Vav2 links EGF-receptor stimulation to RhoA activation [33]. However, siRNA-mediated down-regulation of Vav2 expression had no effect on the activation of RhoA seen on

EGF stimulation (Figure 5). This suggests that, whereas Vav2 is enzymically capable of activating RhoA downstream of EGF stimulation, specificity in the pathway is conferred by the localization of the GEF to the activated receptor, and in turn, of the receptor to the endocytic compartment.

DISCUSSION

We have shown previously that RhoB regulates intracellular traffic of the activated EGF receptor to the lysosome [19]. Here we show that RhoB is itself acutely regulated by EGF-receptor activation, suggesting the existence of an autoregulatory loop linking receptor signalling to receptor traffic. The presence of two separable pools of EGF-stimulated RhoB adds further to the complexity of the system. Although we do not yet know the GEF responsible for the first peak of RhoB activation, use of the Vav2 siRNA allows for studies on the presumed endosomal pool of RhoB activation, in isolation from activation of RhoB at the plasma membrane. This is a subtlety of investigation not possible with the use of dominant-negative RhoB mutants.

This illustrates a general point. Most studies of Rho GTPase signalling pathways have made use of dominant-negative Rho mutants as the primary investigative tool. Rho GTPases, such as RhoA, Rac and Cdc42, regulate a bewildering number of cellular functions, and therefore use of these tools inevitably results in effects outside of the pathway being studied. The ratio of known Rho GEFs to Rho GTPases is at least 3:1. This fanning out of signalling networks at the GEF level suggests that, in many cases, we can expect individual GEFs to act on a more restricted set of pathways than the Rho GTPase(s) they control. We would argue that investigation at the level of GEF activation presents the opportunity for more subtle dissection of Rho GTPase signalling pathways, with the potential to address the pathway under study with minimal perturbation of the broader functions of the Rho GTPases involved. siRNAs are an ideal tool for such studies.

The family of human Rho GEFs contains at least 70 members. Although it is feasible to use an siRNA approach to test each member individually for a fit to a particular signalling pathway, there are three potential shortcuts to such a screen. (i) Overlaying known specificities of Rho GEFs with a dendrogram displaying relatedness of their catalytic domains shows that there is clustering of homology and function [8], and recent work has begun to dissect the molecular basis of this specificity [34]. Hence it is possible, at least in some cases, to identify likely candidates for activation of a particular Rho GTPase, based on the structure of the GEF catalytic domain. (ii) The Rho GEFs are generally large multimodular proteins, with the majority of known interaction domains represented within the regulatory regions of this family. This again provides criteria for narrowing down candidate GEFs in signalling pathways – for example, four human GEFs contain domains involved in calcium signalling, suggesting these are potential candidates for mediating calcium-dependent Rho GTPase pathways. (iii) The Rho GEF family tree clusters into a number of sub-branches. With most branches it is possible to design pan-siRNAs that would specifically target a subgroup of Rho GEFs. In this way it is possible to envisage two-tier screening strategies that would break the whole family down into a more manageable problem.

We would suggest that siRNAs represent a powerful tool in the *in vivo* assignment of Rho GEF function. Further, the ability to target Rho GTPase signalling pathways at the GEF level allows for a more precise dissection of these complex regulatory networks.

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