RhoG is required for both FcγR- and CR3-mediated phagocytosis

George Tzircotis1,+,*1, Vania M. M. Braga2,† and Emmanuelle Caron1
1Centre for Molecular Microbiology and Infection, Faculty of Medicine, Imperial College, London SW7 2AZ, UK
2National Heart and Lung Institute, Faculty of Medicine, Imperial College, London SW7 2AZ, UK

†Present address: Cancer Research Technology Ltd, London EC1V 4AD, UK

*Authors for correspondence (gtzircotis@cancertechnology.com; v.braga@imperial.ac.uk)

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Summary
Phagocytosis is a highly ordered process orchestrated by signalling through Rho GTPases to locally organise the actin cytoskeleton and drive particle uptake. Specific Rho family members that regulate phagocytosis are not known, as the majority of studies have relied on the use of dominant-negative mutants and/or toxins, which can inactivate multiple Rho GTPases. To identify the relevant GTPases for phagocytosis through the Fcγ receptor (FcγR) and complement receptor 3 (CR3), we depleted 20 Rho proteins individually in an RNA interference (RNAi) screen. We find that distinct GTPase subsets are required for actin polymerisation and uptake by macrophages: FcγR-dependent engulfment requires Cdc42 and Rac2 (but not Rac1), whereas CR3 requires RhoA. Surprisingly, RhoG is required for particle uptake through both FcγR and CR3. RhoG has been previously linked to Rac and Cdc42 signalling in different model systems, but not to RhoA. Interestingly, we find that RhoG is also recruited and activated at phagocytic cups downstream of FcγR and CR3, irrespective of their distinct actin structures and mechanisms of internalisation. Thus, the functional links between RhoG and RhoA downstream of CR3-dependent phagocytosis are new and unexpected. Our data suggest a broad role for RhoG in consolidating signals from multiple receptors during phagocytosis.

Key words: CR3, FcγR, Macrophage, Phagocytosis, Small GTPases

Introduction
Engulfment of particulate material by phagocytosis is a vital function of eukaryotic cells and is deployed by organisms as a mechanism for feeding, defense against pathogens and clearance of cellular debris (Desjardins et al., 2005; Underhill and Ozinsky, 2002). Receptor-mediated internalisation occurs through an actin-dependent zippering of membrane around a particle, forming a cup that leads to progressive engulfment (Groves et al., 2008). This process is orchestrated by the Rho GTPase family of proteins through control of actin polymerisation (Groves et al., 2008). Of particular relevance to this study are Fc gamma receptor (FcγR)-mediated phagocytosis, which involves Rac and Cdc42, and complement receptor 3 (CR3)-mediated phagocytosis, which requires Rho (Caron and Hall, 1998).

Several recent reports have contributed to the understanding of GTPase signalling in the early stages of phagocytosis. Rac1, Rac2 and Cdc42 are recruited to FcγR and activated beneath bound particles (Beemiller et al., 2009; Hoppe and Swanson, 2004). Active Rac1 has also been detected in phagocytic cups (Hoppe and Swanson, 2004) and transfection of a dominant-negative Rac1 construct has been reported to inhibit phagocytosis of both IgG- and C3bi-opsonised particles (Cox et al., 1997). Furthermore, a requirement for Rac1 has been shown for phagocytosis of apoptotic cells (Akakura et al., 2004; Nakaya et al., 2008). Taken together, these findings indicate the probable involvement of Rac1 in phagocytic cup formation. Similarly, recruitment and activation of RhoA during internalisation of C3bi-opsonised targets has been shown to require the CR3 cytoplasmic tail (Wiedemann et al., 2006).

Previous studies, however, have generally relied on localisation of transfected constructs and use of dominant-negative GTPases and toxins. Although these approaches have been extensively used and have been very informative, an important caveat is that dominant-negative constructs titrate out upstream regulators (guanine-nucleotide-exchange factors, GEFs) and, similarly, toxins target multiple GTPases (Caron and Hall, 1998; Cox et al., 1997; Massol et al., 1998). As a result, the specific GTPases required for FcγR- and CR3-mediated phagocytosis have not been identified (apart from one example for FcγR) (Jankowski et al., 2008). Here, we undertake a comprehensive RNA interference (RNAi)-based screen to identify the full spectrum of GTPases involved in FcγR and CR3 signalling during phagocytosis.

Results and Discussion
To determine how depletion of individual Rho GTPases interferes with phagocytosis, mouse macrophages phagocytosing either IgG-opsonised (for FcγR) or C3bi-opsonised (for CR3) sheep red blood cells (RBCs) were used as a model. The amount of associated RBCs compared with total RBCs was scored (see Materials and Methods) and different indices were calculated for each RNAi treatment (phagocytic, association indices and the percentage of phagocytosis). Importantly, in both FcγR- and CR3-mediated phagocytosis, there were no statistically significant differences in RBC association to cells (Fig. 1A,D). The latter suggests that RNAi of different small GTPases did not interfere with the availability of receptors at the surface for particle attachment.
For phagocytosis through FcγR, talin silencing was used as a negative control as it is essential only for CR3-mediated phagocytosis (Fig. 1A–C) (Lim et al., 2007). The largest reduction in FcγR percentage phagocytosis was produced by RhoG RNAi (~50%, Fig. 1C). It has been suggested that RhoG can activate Rac1 through an interaction with ELMO2 and DOCK180 (also known as dedicator of cytokinesis protein 1, DOCK1) and lead to increased migration and formation of lamellipodia (Katoh and Negishi, 2003). Interestingly, RhoG, ELMO and DOCK180 have been identified by mass spectrometry to co-precipitate with FcγR and localise at phagocytic cups (Jankowski et al., 2008).

Furthermore, a reduction in the percentage of phagocytosis compared with mock-treated cells was observed following Rac2 silencing, confirming earlier reports of its importance in FcγR-mediated phagocytosis (Jankowski et al., 2008). Although Rac1 has been shown to be in its active GTP-bound state in FcγR phagocytic cups (Beemiller et al., 2009; Hoppe and Swanson, 2004), Rac1 RNAi did not appear to significantly decrease the percentage of phagocytosis in mouse macrophages (Fig. 1C). One interpretation is that depletion of Rac2 interferes with completion of cup closure (Hoppe and Swanson, 2004), preventing particles becoming completely internalised (and therefore not being scored in our system). By contrast, depletion of Rac1 could be compensated for by Rac2 activation at the cups, allowing internalisation to proceed. In addition, our data is supported by the presence of active Rac2 in phagocytic cups (Beemiller et al., 2009; Hoppe et al., 2008; Hoppe and Swanson, 2004) and defective phagocytosis in macrophages derived from Rac2−/− mice (Yamauchi et al., 2004). Interestingly, Rac1−/−, but not Rac2−/−, neutrophils are able to phagocytose non-opsonised Escherichia coli (Koh et al., 2005), indicating that Rac2 is essential for engulfment through other receptors in different cell types.

Although the role of Cdc42 in internalisation of IgG-opsonised particles is well established (Beemiller et al., 2009; Caron and Hall, 1998; Hoppe and Swanson, 2004), Cdc42 RNAi produced only a modest decrease in the levels of phagocytosis (Fig. 1C). As this is likely to be a false-negative result, Cdc42 was included in further phagocytosis assays. Similarly, Rac1 was investigated to exclude the possibility of an artefact.

For CR3-mediated phagocytosis (Fig. 1D–F), talin RNAi induced concomitant decreases in the association and phagocytic indices (Fig. 1D,E), consistent with its crucial role in CR3 activation (Lim et al., 2007). RhoA RNAi, but not RNAi against RhoB or RhoC, strongly reduced the CR3-mediated percentage of phagocytosis (~50% decrease, Fig. 1F). Unexpectedly, a similar reduction in RBC uptake was observed following RhoG RNAi (Fig. 1F), suggesting that RhoG is involved in both FcγR and CR3 signalling.

RhoG plays a role in a variety of cellular processes: engulfment of apoptotic bodies (de Bakker et al., 2004; Nakaya et al., 2006), macrophagocytosis (Ellerbroek et al., 2004), transendothelial cell migration by leukocytes (van Buul et al., 2007), neurite outgrowth and fibroblast migration (Estrach et al., 2002; Katoh et al., 2000). It is interesting to note that RhoG−/− mice show no major growth abnormalities and have largely normal immune system development (Vigorito et al., 2004). However, the susceptibility and response of these mice during infection remains to be explored.

Taken together, our data suggest that FcγR-mediated phagocytosis requires Rac2 and Cdc42; CR3-mediated phagocytosis requires RhoA, whereas RhoG is required for both types of phagocytosis. However, other Rho GTPase family members cannot be formally excluded from participating in particle uptake mediated by FcγR or CR3. A false-negative phenotype can result from issues inherent to RNAi screens, such as different protein half-lives, compensatory effects by other GTPases or inefficient depletion.

In order to validate the GTPases identified here, we next optimised the RNAi conditions (Fig. 2A and Materials and
Methods). Knockdown for each GTPase studied was at least 70% (supplementary material Fig. S1). As controls, and as expected, depletion of talin or RhoA did not play any role in FcγR-dependent phagocytosis (Fig. 2B–D) (Caron and Hall, 1998; Lim et al., 2007). By contrast, Rac1, Rac2 or Cdc42 silencing did not perturb CR3-mediated phagocytosis, providing further evidence of the robustness of this assay (Fig. 2E–G). We confirmed that Rac1 is dispensable for FcγR-mediated phagocytosis, whereas Rac2, Cdc42 and RhoG silencing all significantly reduced IgG-opsonised RBC uptake (Fig. 2B–D). RhoA and RhoG depletion interfered with the uptake of C3bi-opsonised particles (Fig. 2G). Silencing of RhoG had no effect on the expression levels of Rac2, Cdc42 or RhoA, indicating that RhoG function in both types of phagocytosis was specific (data not shown). Taken together, these results strongly support the main findings of the primary screen, that is, that signalling downstream of FcγR and CR3 require different GTPases but both require RhoG. Furthermore, we exclude the participation of Rac1, RhoB and RhoC as regulators of particle uptake through FcγR- or CR3-mediated phagocytosis.

Fig. 2. RhoG is necessary for both FcγR- and CR3-mediated phagocytosis. (A) 3774 A.1 macrophages were transfected with RNAi duplexes against talin and the indicated Rho GTPases. Cells were then lysed and analysed by western blotting (WB) using the indicated anti-GTPase antibody, with actin as a loading control. Blots are representative of at least three independent experiments. (B–G) Phagocytic assays showing the association index (B,E), phagocytic index (C,F) and percentage of phagocytosis (D,G) for FcγR-mediated (B–D) or CR3-mediated (E–G) phagocytosis following RNAi against the indicated Rho GTPase. Blind scoring was performed for over 200 cells in each of at least three independent experiments. Error bars represent standard error. *P<0.05, ***P<0.01.

An important early event in GTPase signalling downstream of FcγR and CR3 is actin recruitment, which drives the formation of phagocytic cups (Groves et al., 2008; van Zon et al., 2009). We hypothesised that depletion of the GTPases validated in our screen would interfere with F-actin recruitment to phagocytic cups (hereafter referred to as actin cups). To address this possibility, the percentage of attached, opsonised RBCs colocalising with F-actin in phagocytic cups was quantified following RNAi of different GTPases. Representative images clearly illustrate the raised F-actin-rich cups typical of FcγR-mediated engulfment (Fig. 3A) and the much less prominent cups formed during CR3-mediated engulfment (Fig. 3C). Treatment with cytochalasin D severely perturbed actin polymerisation in both FcγR and CR3 phagocytic cups, as shown previously (Groves et al., 2008).

For phagocytosis of IgG-opsonised RBCs, Rac2s and Cdc42 silencing reduced the number of cups containing F-actin compared with the number following RNAi against talin (45% and 25%, respectively, Fig. 3B). In addition, depletion of RhoA decreased actin cup formation by 50%, whereas talin RNAi decreased this process by 70% (Fig. 3D). Interestingly, macrophages depleted of RhoG showed an ∼65% reduction in actin cup formation for both FcγR- and CR3-mediated phagocytosis (Fig. 3B,D). Hence, for F-actin recruitment during phagocytosis, we demonstrate that Rac2 and Cdc42 are required downstream of FcγR, RhoA is required downstream of CR3, and RhoG is necessary downstream of both receptors.

We next asked whether (i) RhoG is specifically recruited to phagosomes and (ii) RhoG is activated downstream of both FcγR and CR3. We examined RhoG recruitment to phagocytic cups by co-transfection of wild-type (WT) RhoG and either WT FcγRIIa or CR3. Upon challenge with opsonised RBCs (IgG or C3bi), RhoG colocalised with actin cups (Fig. 4A). No significant RhoG recruitment to sites of bound RBCs was observed in cells transfected with signalling-deficient mutants, FcγRIIa lacking the cytoplasmic tail, FcγRIIa–A239 (van Zon et al., 2009) and CR3 with a triple threonine to alanine replacement in the integrin β2 chain (zMβ2-T758A/T759A/T760A, CR3-TTT-AAA) (Wiedemann et al., 2006). Therefore, the intact cytoplasmic tails of FcγRIIa and CR3 are essential for recruitment of RhoG to phagocytic cups.

To address RhoG activation during phagocytosis, we used ELMO-T625–GFP, a mutant of ELMO1 that lacks the DOCK180-binding domain and interacts exclusively with active RhoG (Katoh and Negishi, 2003). Upon co-transfection of ELMO-T625–GFP with WT FcγRIIa or WT CR3, prominent recruitment of RhoG-GTP was observed at sites of particle binding, where it colocalised with F-actin (Fig. 4B). Thus, RhoG is recruited (Fig. 4A) and activated (Fig. 4B) at the forming phagosome.

To determine whether RhoG activation is specifically mediated by signalling from FcγR and CR3, we scored recruitment of ELMO-T625–GFP to WT receptors and two signalling-deficient receptor mutants: CR3-TTT-AAA and FcγRIIa-Y282F/Y298F, which contains phenylalanine replacements in two tyrosine residues crucial for the initiation of signalling and actin polymerisation (Fig. 4C) (Mitchell et al., 1994) (Fig. 4D). As a control, recruitment of GFP alone to WT receptors was also measured. RhoG-GTP recruitment to both signalling-deficient mutants was significantly impaired compared with WT versions of each receptor (Fig. 4C,D). We conclude that tyrosine residues 282 and 298 of the FcγRIIa cytoplasmic tail and threonines 758–760 of RhoG.
the CR3 integrin β2 chain are required for the local activation of RhoG during phagocytosis.

Interestingly, threonine residues 758–760 of the integrin β2 cytoplasmic tail are also required for recruitment of RhoA to CR3 (Wiedemann et al., 2006). It is possible that this domain is crucial for the formation of a scaffold complex that recruits both RhoA and RhoG. Alternatively, RhoG might be required for RhoA activation. In future studies, it will be interesting to dissect the crosstalk between RhoG and other Rho GTPases during particle uptake.

In conclusion, our RNAi screen demonstrates the specific activation of RhoG during FcγR- and CR3-mediated phagocytosis and its requirement for actin cup formation. Surprisingly, we find that RhoG is also recruited to phagocytic cups and activated during CR3-mediated phagocytosis. This is the most striking and novel finding, as there is no previous evidence linking RhoA signalling with RhoG. Our data imply that two very distinct mechanisms of phagocytosis, with different actin structures, receptors and signalling proteins can share a few common partners. RhoG is one of these links, as it can crosstalk with RhoA or Rac2-Cdc42, depending on the phagocytic receptor.

Fig. 3. RhoG is required for actin polymerisation during FcγR- and CR3-mediated phagocytosis. J774 A.1 macrophages transfected with RNAi against RhoG or incubated in the presence or absence of 0.5 μM cytochalasin D (CytD) were challenged with IgG- (A) or C3bi-opsonised (C) RBCs. Cells were then fixed, and opsonised RBCs were labelled with IgG (red) and F-actin was visualised using phalloidin (green). Representative projections of confocal microscopy sections are shown. Arrows indicate typical F-actin morphology in phagocytic cups. (B,D) Quantification of actin cup formation for FcγR-(B) or CR3-mediated (D) phagocytosis, expressed as the percentage of the actin cups found in the mock transfection and negative control. For each treatment, over 200 cells were blind scored. Error bars represent standard error from at least three independent experiments. *P<0.05, ***P<0.01. Scale bars: 10 μm.

Fig. 4. RhoG-GTP localises to wild-type FcγR and wild-type CR3 phagocytic cups but not to signalling-deficient receptor mutants. COS-7 cells were transfected with the indicated constructs and phagocytic receptors and were challenged with IgG- or C3bi-opsonised RBCs. Cells were fixed and processed for immunofluorescence with anti-IgG antibody to label opsonised RBCs (white), phalloidin (blue) and either anti-CD11b antibody or anti-FcγR antibody, as appropriate (red). (A) Co-transfection of GFP–RhoG and either wild-type (WT) FcγR or CR3 or their signalling-deficient mutants (FcγR-Δ239 or CR3-TTT-AAA). (B) Co-transfection of the RhoG-GTP reporter ELMO-T625–GFP (green) and WT FcγR (top) or CR3 (bottom). (C,D) GFP or ELMO-T625–GFP were transfected together with the indicated WT receptors or signalling-deficient mutants (FcγR-Y298F/Y298F or CR3-TTT-AAA). Phagocytic cups for over 20 cells were scored blind for each transfection in at least three independent experiments. Data are expressed as the percentage of recruitment to RBCs. Error bars correspond to the standard error. *P<0.05, ***P<0.01. Scale bars: 5 μm.
In physiological conditions, microbes coming into contact with professional phagocytes will probably be coated with multiple opsonising molecules leading to activation of a variety of signalling pathways (Underhill and Ozinsky, 2002). Subversion of RhoG activity during cell invasion by bacterial pathogens, such as Salmonella enterica (Patel and Galan, 2006) and pathogenic Yersinia species (Mohammadi and Isberg, 2009; Roppenser et al., 2009), reveals a fundamental role for RhoG in phagocytosis by different receptors. Taken together with our data, a pattern emerges for the key participation of RhoG in FcγR- and CR3-mediated internalisation, as well as downstream of other phagocytic receptors. The participation of RhoG in the engulfment of apoptotic cells and its remarkable broad range of functions raise the possibility that RhoG is a key node of phagocytic signalling convergence.

Materials and Methods

Cells

COS-7 cells and J774 A.1 mouse macrophages were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen).

Antibodies

Rabbit polyclonal antibodies against the following proteins were used: Cdc42 (Cell Signaling Technology), Rac2 (Santa Cruz Biotechnology), Rac1 (Cytoskeleton) and actin (Sigma-Aldrich). Monoclonal antibodies used were against: RhoA (clone 67B9, Cell Signaling Technology), RhoG (clone 1F3 B3 E5, Millipore), talin (clone 3D4, Sigma-Aldrich), FcyRIIa (clone IV.3, Abcam) and CD11b (clone M1/70, Serotec). Horseradish peroxidase (HRP)-conjugated anti-mouse-IgG was purchased from GE Healthcare and HRP-conjugated anti-rabbit-IgG from Santa Cruz Biotechnology. Alexa Fluora-conjugated secondary antibodies and phalloidin were purchased from Invitrogen.

Constructs and transfections

pRK5-FcγRIIa, pRK5-CD11b, pRK5-CD18 (Caron and Hall, 1998), pRK5-FcγRIIa-Y227F/Y229F (Cou goule et al., 2004), pRK5-FcγR-IIa-A239 (van Zon et al., 2009) and pRK5-CD18-T758A/T759A/T760A (Wiedemann et al., 2006) have been previously described. pEGFP-C1-ELMO1-T625 (Handa et al., 2007) was a gift from Chihiro Sasaki (University of Tokyo, Japan) and pEGFP-C1-RhoG (Patel and Galan, 2006) was a gift from Jayesh Patel (Yale University, New Haven, CT). COS-7 cells were transfected with plasmids using an Amxa nucleofector and nucleofector cell line kit R according to the manufacturer’s instructions. For phagocytosis assays, transfected cells were seeded onto glass coverslips in 24-well plates at a density of 15,000 cells per coverslip and incubated at 37°C.

RNAi

For the primary screen of Rho GTPases, 50,000 J774 A.1 mouse macrophages that were internalised (labelled only with Alexa Fluor 488) and those particles that were bound but not internalised (labelled with both Alexa Fluor 555 and Alexa Fluor 488). The association index was calculated as the number of all bound RBCs (external and internal) divided by the number of cells scored, whereas the phagocytic index was calculated using only the number of internalised RBCs divided by the number of cells scored. The amount of particles (associated or internalised) in controls was arbitrarily set as 100 and values were calculated relative to controls. The percentage of phagocytosis was then calculated as the phagocytic index divided by the association index, multiplied by 100 (Lim et al., 2007). The percentage recruitment of actin, GFP alone or ELMO-T625–GFP was calculated as the number of associated RBCs colocalising with different exogenous proteins divided by the total number of associated RBCs, multiplied by 100.

Immunofluorescence and microscopy

For immunofluorescence, differential staining to distinguish between the internalised and total bound RBCs was used (Lim et al., 2007; van Zon et al., 2009). Briefly, following phagocytic challenge, external RBCs were labelled with Alexa-Fluor-555-conjugated anti-rabbit-IgG at 4°C for 5 minutes. Cells were then fixed, permeabised and all particles labelled with Alexa-Fluor-488-conjugated anti-rabbit-IgG at room temperature for 30 minutes. Thus, external RBCs were identified by co-labelling with Alexa Fluor 555 and Alexa Fluor 488 and internalised RBCs by exclusive labelling with Alexa Fluor 488.

For receptor expression levels, transfected cells were fixed, permeabised and labelled with anti-FcγRIIa or anti-CD11b antibody followed by Alexa-Fluor-555-conjugated anti-mouse-IgG antibody. RBCs were visualised with Alexa-Fluor-405-conjugated anti-rabbit-IgG antibody and F-actin with Alexa Fluor-647-conjugated phalloidin. Sequential z-series image stacks were acquired on a Zeiss LSM-510 confocal microscope using a step size of 0.4 μm, processed using Zeiss LSM-510 software and shown as maximum projections.

Scoring for each transfection or treatment, cells were blind-scored for the number of particles that were internalised (labelled only with Alexa Fluor 488) and those particles that were bound but not internalised (labelled with both Alexa Fluor 555 and Alexa Fluor 488). The association index was calculated as the number of all bound RBCs (external and internal) divided by the number of cells scored, whereas the phagocytic index was calculated using only the number of internalised RBCs divided by the number of cells scored. The amount of particles (associated or internalised) in controls was arbitrarily set as 100 and values were calculated relative to controls. The percentage of phagocytosis was then calculated as the phagocytic index divided by the association index, multiplied by 100 (Lim et al., 2007). The percentage recruitment of actin, GFP alone or ELMO-T625–GFP was calculated as the number of associated RBCs colocalising with different exogenous proteins divided by the total number of associated RBCs, multiplied by 100.

Statistical significance of the RNAi, and the treatments in the phagocytic and actin cup formation assays, was calculated using the Student’s t-test compared with control RNAi for FcγR-mediated phagocytosis and Rac1 RNAi for CR3-mediated phagocytosis.

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