Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration

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Summary

Rac GTPases are activated by extracellular stimuli and contribute to cellular responses including cytoskeletal changes and cell migration. Dominant-negative Rac1 has been used to implicate Rac GTPases in these responses, but which of the three mammalian Rac isoforms it inhibits is not known. We show that mouse bone marrow-derived macrophages express Rac1, low levels of Rac2 but not Rac3. As Rac1-null mice die early in development, we have used mice with a loxP-flanked allele of *Rac1* and the type I interferon-inducible Mx1-Cre transgene to address for the first time the specific role of Rac1 in cell motility. Bone marrow-derived macrophages isolated from mice treated with polyIC to induce interferon lack detectable Rac1, and there is no compensatory increase in Rac2 or Cdc42 expression. Rac1-deficient macrophages have an altered morphology: they are significantly more elongated than control cells and have a reduced adhesive area. Re-expression of Rac1 reverts the morphology to that of control cells. Loss of Rac1 reduces but does not completely prevent membrane ruffling in response to CSF-1. However, Rac1-deficient macrophages show normal migration and chemotaxis. Thus in macrophages Rac1 is primarily responsible for regulating cell morphology, contributes to membrane ruffling, but is not required for migration.

Movies available online

Key words: Rac1, Cell spreading, GTPase, Macrophage

Introduction

The Rho family of small GTPases includes Rac, Rho and Cdc42 proteins, which act as morphological switches cycling between a GTP-bound (active) and GDP-bound (inactive) state to relay messages from cell surface receptors to the cytoskeleton. The GTP/GDP binding cycle is regulated by the interaction of Rho family GTPases with guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs catalyse the exchange of GDP for GTP, whereas GAPs enhance the intrinsic GTPase activity thus rendering the proteins inactive (Symons and Settleman, 2000; Van Aelst and D'Souza-Schorey, 1997)

Rac proteins play a central role in cell migration by inducing the extension of lamellipodia, thin sheet-like structures that push the plasma membrane outward through actin polymerisation. Lamellipodia are found at the leading edge of motile cells, and Rac proteins have been shown to be required for cell migration in a wide variety of cell types (Ridley, 2001). There are three isoforms of Rac (1, 2 and 3) in mammals, but little is known about the relative contributions of each isoform to Rac-dependent responses. All three proteins are highly homologous with the primary differences in sequence concentrated in the carboxy-terminal end of the proteins (Didsbury et al., 1989; Haataja et al., 1997). Each isoform is highly conserved between species: for example murine Rac1 differs by only one amino acid from human RAC1 (Didsbury et al., 1989; Shirsat et al., 1990). Rac1 is the most extensively studied isoform and is ubiquitously expressed. There is a splice variant of Rac1 (Rac1b) but little is known of its expression pattern other than in epithelial cells (Jordan et al., 1999). In contrast, Rac2 appears to be expressed only in cells of haematopoietic origin (Didsbury et al., 1989). Rac3 is expressed in a number of tissue types and is highly expressed in the brain (Haataja et al., 1997; Malosio et al., 1997).

Previous work on the role of Rac proteins in growth factor-, cytokine- and adhesion-stimulated lamellipodium extension and/or membrane ruffling has primarily been based on the use of constitutively active or dominant negative mutants (Allen et al., 1997; Ridley and Hall, 1992). Microinjection of both activated Rac1 and Rac2 into fibroblasts induces membrane ruffling (Ridley et al., 1992; Xu et al., 1994). Conversely, dominant negative Rac1 (N17Rac1) inhibits lamellipodial formation, membrane ruffling and cell migration in a number of cell types, including macrophage cell lines (Allen et al., 1997; Cox et al., 1997), although it does not inhibit membrane ruffling in dendritic cells (West et al., 2000). However, it is not known whether N17Rac1 inhibits only Rac1 activation or also Rac2, Rac3 and possibly other closely related Rho family GTPases. To address the specific functions of each Rac isoform, Rac1- and Rac2-null mice were generated. Haematopoietic stem cells, neutrophils, T cells and B cells derived from Rac2-null mice are defective in cell migration, chemotaxis and cell adhesion (Croker et al., 2002a; Roberts et al., 1999; Yang et al., 2001). However, Rac1-null mice die early

in development because of defects in formation of the three germ layers at gastrulation (Sugihara et al., 1998), so it has not so far been possible to study the motile behaviour of Rac1-deficient cells in detail.

We have used bone marrow-derived macrophages from mice with a conditional knockout of Rac1 to study the specific contribution of Rac1 to motility in cells that express two Rac isoforms, Rac1 and Rac2. Surprisingly, we find that Rac1 is not required for macrophage migration nor is it essential for the formation of membrane ruffles in response to CSF-1. In contrast, Rac1-deficient cells have a marked change in cell morphology suggesting that Rac1 contributes primarily to cell spreading.

Materials and Methods

Isolation and culture of bone marrow-derived macrophages (BMMs)

The generation of mice bearing a conditional loxP-flanked allele of *Rac1*, *Rac1*^{flox}, is described elsewhere (Walmsley et al., 2003). This allele contains loxP sites in intron 3 and intron 5 of *Rac1*, flanking exons 4 and 5, such that recombination between the loxP sites catalysed by the Cre recombinase results in deletion of the intervening region and hence elimination of Rac1 protein expression. Mice bearing the *Rac1*^{flox} allele on a 129/Sv;C57BL/6 segregating genetic background were crossed to mice containing the type I interferon-inducible Mx1-Cre transgene on a C57BL/6 background (Kuhn et al., 1995), to generate *Rac1*$^{flox/flox}$Mx1-Cre mice. To induce expression of the Mx1-Cre transgene, the mice were given a 150 μl intraperitoneal injection of synthetic double-stranded RNA polyinosinic-polycytidylic acid (polyIC, 2 mg/ml) on three separate occasions at 2-day intervals. DNA isolation and Southern blotting was performed by standard techniques. To generate BMMs, mice were left for at least 2 days after the final injection of polyIC before culling and harvesting of the bone marrow as previously described (Leverrier and Ridley, 2001). BMMs were isolated from mice whose genotypes were *Rac1*$^{+/+}$ (Wt), *Rac1*$^{+/+}$Mx1-Cre (WtCre) and *Rac1*$^{flox/flox}$Mx1-Cre (Rac1$^{-/-}$). Cells were seeded at 2×10^5 cells/cm^2 in 25 cm^2 tissue culture flasks (Nunc, Fisher Scientific, UK) in macrophage growth medium consisting of RPMI-1640 (Gibco, Invitrogen Corporation, UK), 1 mM sodium pyruvate (Gibco), 1× nonessential amino acids (Gibco), 0.2 mM 2-mercaptoethanol (Sigma, UK), and 10% heat inactivated foetal bovine serum (Sigma) supplemented with 30% L cell-conditioned medium (L-cell) as a source of CSF-1. After 3 days, non-adherent cells were collected, and either cryogenically stored in macrophage growth medium containing 10% dimethyl sulphoxide (DMSO, Sigma) or seeded at 10^5 cells/ml in bacteriological plates (Falcon), and grown for 5 days before use. Cells were then harvested using Versene (1:5000), centrifuged at 1000 ***g*** and resuspended in macrophage growth medium. Freezing did not detectably modify the characteristics or the behaviour of the macrophages that were obtained. All the results described here are from cells that had been cultured for no more than a total of 2 weeks. To control for animal-to-animal variation the results presented here are derived from five different BMM isolations.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and then permeabilised with 0.2% Triton X-100 in PBS for 5 minutes. For actin staining alone cells were then incubated with TRITC-conjugated phalloidin (Sigma) diluted in PBS containing 20% goat serum for 1 hour at room temperature. Following incubation, cells were washed six times in PBS. To detect the Flag tag, cells were pre-blocked with PBS containing 20% goat serum for 30 minutes prior to a 1 hour incubation with a 1:200 dilution of a mouse anti-Flag antibody (Sigma) in 20% goat serum/PBS. Cells were then washed six times in PBS and incubated with a 1:400 dilution of FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PS) and TRITC-conjugated phalloidin. Images of cells were obtained using a Zeiss LSM510 confocal laser-scanning microscope (Welwyn Garden City, UK), using the accompanying LSM 510 software, and were processed in Adobe Photoshop 4.0.

Quantification of cell adhesive area and elongation ratio

For analysis of cell adhesive area and elongation ratio cells were seeded on 13-mm coverslips at a density of 2×10^4 cells/ml and incubated for 24 hours. Cells were then either incubated in macrophage growth medium or in medium lacking L-cell conditioned medium (macrophage starve medium) for 18 hours. Where indicated, cells were re-stimulated with CSF-1 (30 ng/ml; R and D systems, Abingdon, UK) for 5 minutes and then fixed. All the cells were stained for F-actin as described above. To analyse cell adhesive area and elongation, digitised images acquired by confocal microscopy were analysed with Image Pro Plus. Cell elongation was calculated as described by Dunn and Brown (Dunn and Brown, 1986) as the ratio of the long axis of the cell to the shortest axis of the cell. Data are presented as means±s.e.m.. Student's *t*-test was used to compare differences between groups. Statistical significance was accepted for $P<0.05$.

Microinjection

Cells for microinjection were seeded in 15 mm wells at 10^4 cells per well on 13 mm circular glass coverslips and incubated for 24 hours in macrophage growth medium followed by a 16 hour macrophage starve medium. Cells were microinjected with 100 ng/μl pCMV-Flag-placental-N17Cdc42, pCMV-Flag-N17Rac1 or pCMV-Flag-Rac1. Three hours after microinjection, cells were stimulated with CSF-1 (30 ng/ml; R and D systems, Abingdon, UK) for 5 minutes, then fixed with 4% paraformaldehyde, and stained for Flag-tagged protein and F-actin as described above. Images of the CSF-1-induced ruffles and total adhesive area were generated using confocal microscopy. Total adhesive area and elongation ratios were calculated as above.

Quantification of ruffling

A ruffle was defined as an F-actin-rich undulating membrane protrusion on the dorsal surface of a cell using fluorescence microscopy. The extent of CSF-1-induced ruffling was quantified as follows [modified from Cox et al. (Cox et al., 1997)]: 0 = no ruffles, 1 = isolated areas of ruffling covering no more than 50% of the dorsal surface, and 2 = extensive ruffling covering more than 50% of the dorsal surface.

Time-lapse microscopy

To study random migration, cells were seeded at a density of 2×10^4 cells/ml on 2 cm dishes (Nunc) in macrophage growth medium and allowed to adhere for 6 hours prior to being placed on the microscope stage. Cell images were collected with a KPM1E/K-S10 CCD camera (Hitachi Denshi, Japan) every 5 minutes for 20 hours using Tempus software (Kinetic Imaging Ltd, Liverpool, UK). To study chemotaxis, cells were seeded on acid washed 22×22 mm coverslips at a density of 2×10^4 cells/ml in macrophage growth medium and incubated overnight. Following incubation cells were starved of CSF-1 in macrophage starve medium for 8 hours. The coverslips were then mounted onto Dunn chemotaxis chambers as previously described (Allen et al., 1998) using recombinant murine CSF-1 (30 ng/ml) as the chemoattractant. To study random

migration on glass both wells of the Dunn chemotaxis chamber were filled with medium supplemented with CSF-1. Cell images were collected every 10 minutes as described above. Subsequently all the acquired time-lapse sequences were displayed as a movie and each cell in the first frame was tracked for the whole of the time-lapse sequence using Motion Analysis software (Kinetic Imaging Ltd, Liverpool, UK). This resulted in the generation of a sequence of position co-ordinates relating to each cell in each frame. Mathematical analysis was then carried out using Mathematica 6.0 (Wolfram Research Institute) workbooks (Allen et al., 1998) and Excel 2000 (Microsoft).

Immunoblotting

BMMs were lysed in 2× GSB (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue and 2% 14.3 M β-mercaptoethanol). Recombinant Rac1 and Rac2 proteins were a kind gift from Elena Prigmore. They were purified as GST-fusion proteins from *E. coli* and cleaved from GST using thrombin as previously described (Ridley et al., 1992). Protein concentrations were determined using BioRad protein assay reagent. BMM lysate and recombinant Rac1 or Rac2 protein were electrophoresed on 10% SDS-polyacrylamide gels then transferred to nitrocellulose membranes (Schleicher and Schuell). Membranes were blocked in 5% non-fat dried milk in PBS then incubated for 16 hours at 4°C with mouse anti-Rac1 (Upstate Biotechnology, NY, USA), rabbit anti-Rac2 (kind gift of Gary Bokoch) or mouse anti-tubulin (Sigma, UK) antibodies. Membranes were then incubated for 1 hour at room temperature with a 1:2000 dilution of horseradish-peroxidase-conjugated donkey anti-rabbit or anti-mouse antibody in 0.5% non-fat milk in PBS (Amersham Pharmacia, Little Chalfont, UK). Blots were developed by enhanced chemiluminescence (ECL, Amersham Pharmacia).

RT-PCR

To elucidate the expression profile of Rac isoforms in CSF-1-dependent bone marrow macrophages, RT-PCR was performed with isoform-specific primers derived from mouse cDNA sequences (Rac1 sense CCCAATACTCCTATCATCCTCG, Rac1 antisense CAGCAGGCATTTTCTCTTCC; Rac2 sense CCAGCACCCCCATCATCCTGG, Rac2 antisense GGGGCGCTTCTGCTGTCGTGTG; Rac3 sense CACACACCCATCCTTCTGGTG, Rac3 antisense CAGTGCACTTCTTGCCTGGC). Total RNA was isolated from growing WtCre cells using Trizol (Invitrogen) extraction and isopropanol precipitation. Prior to cDNA synthesis the RNA was treated with DNAse I (Invitrogen) to remove any residual genomic DNA. First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR conditions were 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds. Rac1/2/3-specific primers produced amplicons of 240 bp, 240 bp, and 250 bp, respectively.

Macrophage adhesion assay

Cells to be used in the adhesion assays were removed from bacteriological plates and washed twice in serum-free RPMI (Gibco). 2×10^5 cells were then seeded on 2 cm dishes (Nunc) that were untreated or pre-coated with 10 μg/ml fibronectin (Sigma). After 60 minutes the dishes were washed twice with PBS and the cells were then incubated in medium supplemented with 250 μg/ml methylthiazoletetrazolium (MTT) for 4 hours. The cells were then solubilised for 1 hour using SDS to a final concentration of 3.5%. Finally, the absorbance at 595 nm was quantified using a Bio-Rad SmartSpec 3000 spectrophotometer. Experiments were performed in duplicate and standards of incremental cell densities and adhesion time were used as controls.

Cdc42 activation assay

The Cdc42 activation assay described here was based on the Rac activation assay method provided by Upstate Biotechnology (www.upstatebiotech.com). The WASP p21-binding domain (PBD) in pGEX (a generous gift from David Sacks) was expressed in *E. coli* strain XL1-Blue as a fusion protein. The GST fusion protein was purified from bacterial cell lysates with gluatathione-Sepharose beads and stored at –70°C in 10 mM Tris pH 8, 1 mM DTT, 100 mM NaCl and 50% glycerol. BMM (5×10^5 cells per time point) were washed with cold PBS and lysed in 1× lysis buffer followed by immediate centrifugation at 13,000 ***g*** for 3 minutes. A small proportion of the lysate was removed for protein concentration assay (BioRad) and western blot analysis of total Cdc42 levels. Cleared lysates were then incubated for 45 minutes with pre-washed GST-WASP-PBD beads at 4°C. The beads were pelleted by centrifugation at 13,000 ***g*** for 5 seconds and washed three times with 1× lysis buffer. The beads were finally resuspended in 30 μl of 2× gel sample buffer. Samples were separated by 12.5% SDS-PAGE and western blotted with anti-Cdc42 antibodies (Santa Cruz).

Results

Mouse BMMs express Rac1 and Rac2 but not Rac3

Previous work in the mouse macrophage Bac1 cell line has indicated that Rac proteins are required for macrophage migration (Allen et al., 1997), but the role of each Rac isoform has not been investigated, as dominant negative Rac1 (N17Rac1) is not known to be isoform-specific. Of the three known mammalian Rac isoforms, Rac1 is expressed and rapidly activated by CSF-1 in B-lymphocytes and BMMs (Grill and Schrader, 2002) (Elena Prigmore and A.J.R. unpublished), and it has previously been reported that Rac2 and Rac3 are expressed in HL-60 cells, a human myeloid cell line (Didsbury et al., 1989; Haataja et al., 1997). As there is no antibody available that specifically recognises Rac3 we performed RT-PCR on mRNA isolated from control BMMs to determine which Rac isoforms were expressed in BMMs. Primers specific to each Rac isoform were designed (see Materials and Methods). The primers generated were all isoform specific as demonstrated by PCR reactions using plasmids containing Rac1, Rac2 or Rac3 cDNA as controls (Fig. 1A). The Rac1 and Rac2 primer sets generated easily detectable amplicons in a RT-PCR reaction of BMM cDNA. However, even following 30 cycles of PCR a Rac3 amplicon was not detected in BMM cDNA (Fig. 1B). This result was confirmed by repeating the amplification using primers designed to the Rac3 3′-UTR (data not shown). An alternative splice variant of Rac1, Rac1b, includes a 19-amino acid insertion (Jordan et al., 1999) in a region spanned by the Rac1 primers. However, as we observed only a single amplicon using the Rac1 primer set it appears that BMMs do not express Rac1b. Having established that BMMs express Rac1 and Rac2 the relative expression of these Rac isoforms was compared in growing BMMs using recombinant Rac1 and Rac2 proteins of known concentration (Fig. 1C,D). These results indicate that Rac1 is expressed at approximately threefold higher levels than Rac2 in BMMs. Antibodies to Rac2 consistently gave a weak signal in BMM whole cell lysates, even though they produced a strong signal in human neutrophil lysates (data not shown), consistent with the observation that human neutrophils express predominantly Rac2 and only low levels of Rac1 (Heyworth et al., 1994). Recently it has been reported that murine neutrophils express similar amounts of Rac1 and Rac2 suggesting that Rac

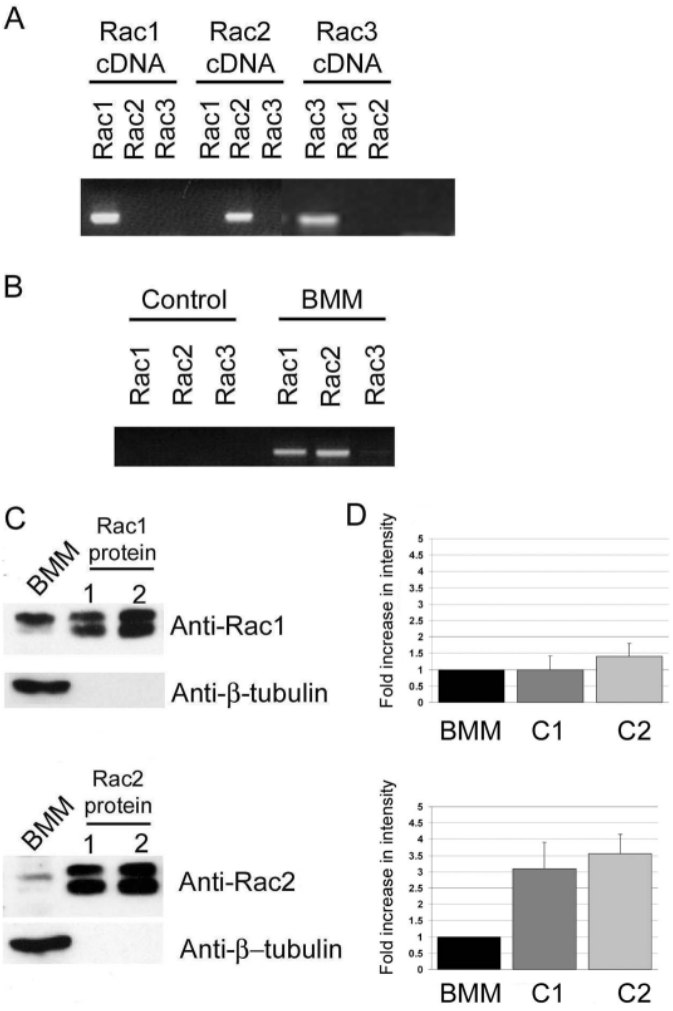

Fig. 1. Rac isoform expression in bone marrow-derived macrophages (BMMs). (A) RT-PCR to establish primer specificity. RT-PCR was performed on plasmid DNA containing *Rac*1, *Rac*2 or *Rac*3 cDNA using the *Rac*1/2/3 isoform-specific primers as described in Materials and Methods. (B) RT-PCR to establish *Rac* isoform expression in BMMs. RT-PCR was performed on cDNA derived from Wt BMM RNA using the same primer sets as in A). As a control for the presence of genomic DNA in the cDNA preparation RT-PCR was also carried out on cDNA prepared in the absence of reverse transcriptase. (C) Analysis of Rac1 and Rac2 protein expression levels in BMMs. Lysate from Wt BMMs and recombinant Rac1 (top) and Rac2 (bottom) protein (lanes 1 and 2; 1.5 and 2.5 ng, respectively) were resolved by SDS-PAGE, western blotted and probed with anti-Rac1 (top) or anti-Rac2 (bottom) antibody. Blots were re-probed with anti-β-tubulin antibody. (D) Bands on autoradiographs of three western blots similar to those in C were quantified using Kinetic Imaging software. The level of Rac1 or Rac2 protein in BMMs was compared with the level of recombinant Rac1 or Rac2 (lane 1, 1.5 ng).

isoform expression levels may vary between species or during differentiation (Li et al., 2002).

Generation and characterisation of Rac1-deficient macrophages

To elucidate the role of Rac1 in the migratory and cytoskeletal responses of macrophages, we generated Rac1-deficient BMMs. Since the disruption of the *Rac1* gene in mice by conventional gene targeting methodology causes early embryonic lethality (Sugihara et al., 1998), we chose to use mice bearing a conditional loxP-flanked allele of *Rac1* termed *Rac1*^{flox} (Walmsley et al., 2003). The *Rac1*^{flox} allele contains two loxP sites flanking exons 4 and 5, such that recombination between the loxP sites, catalysed by the Cre recombinase, causes deletion of these exons and abrogates expression of the Rac1 protein. To allow inducible deletion of the *Rac1* gene, we crossed these mice to ones bearing the type I interferon-inducible Mx1-Cre transgene (Kuhn et al., 1995). Injection of polyIC (which causes release of type I interferons) into *Rac1*$^{flox/flox}$Mx1-Cre mice caused efficient deletion of the *Rac1* gene in the bone marrow (Fig. 2A). This marrow was then used to generate macrophages hereafter referred to as Rac1$^{-/-}$ BMMs. In addition, as controls, we also grew macrophages from the bone marrow of *Rac1*$^{+/+}$ (Wt) and *Rac1*$^{+/+}$Mx1-Cre (WtCre) mice that had been injected with polyIC. In none of the assays described here did the injection of polyIC into the donor mice detectably change the characteristics or behaviour of the macrophages obtained from them compared to macrophages derived from uninjected mice (data not shown). It has been reported that high levels of Cre recombinase expression can result in chromosomal rearrangements (Schmidt et al., 2000), therefore we compared Wt and WtCre BMMs as controls. However, in none of the assays used here did we detect any differences between Wt and WtCre macrophages. Furthermore, there was no detectable difference in BMM maturation between control (Wt and WtCre) and Rac1$^{-/-}$ populations as measured by flow cytometric analysis of F4/80 [a surface marker for differentiated macrophages (Leverrier and Ridley, 2001)] and c-fms (CSF-1 receptor) expression (data not shown). There was no detectable expression of Rac1 protein in Rac1$^{-/-}$ BMMs (Fig. 2B). Furthermore, the loss of Rac1 was not compensated for by an upregulation of Rac2 (Fig. 2B) or Cdc42 expression (data not shown).

It has previously been shown that in Rac2$^{-/-}$ haematopoietic progenitor cells Rac1 and Cdc42 expression levels were unaltered but that their basal level of activity was increased approximately three- and 20-fold respectively (Yang et al., 2001). Moreover, recent analysis of Rac2$^{-/-}$ neutrophils has demonstrated a threefold increase in Rac1 activity (Li et al., 2002), although these cells still exhibited severe migratory defects. We therefore compared the activity of Cdc42 in growing Rac1$^{-/-}$ and Wt cells (Fig. 2C). There was no significant difference in the level of Cdc42 activity in growing Wt and Rac1$^{-/-}$ BMMs or between control and Rac1$^{-/-}$ cells during CSF-1 stimulation. Indeed, CSF-1 starvation only slightly reduced Cdc42 activity in both populations and re-stimulation with CSF-1 did not significantly increase Cdc42 activity at 2 minutes, or other time points, even though CSF-1-activated ERK phosphorylation was detected in these cells (Fig. 2D and unpublished data). We therefore conclude that there was no difference in Cdc42 activity in Rac1$^{-/-}$ BMMs compared to control BMMs. In Rac2$^{-/-}$ haematopoietic progenitor cells increased Cdc42 activity was accompanied by a constitutive and extensive increase in filopodial extension (Yang et al., 2001), whereas few filopodia were observed on Rac1$^{-/-}$ BMMs (Fig. 3A). We were unable to quantify Rac2

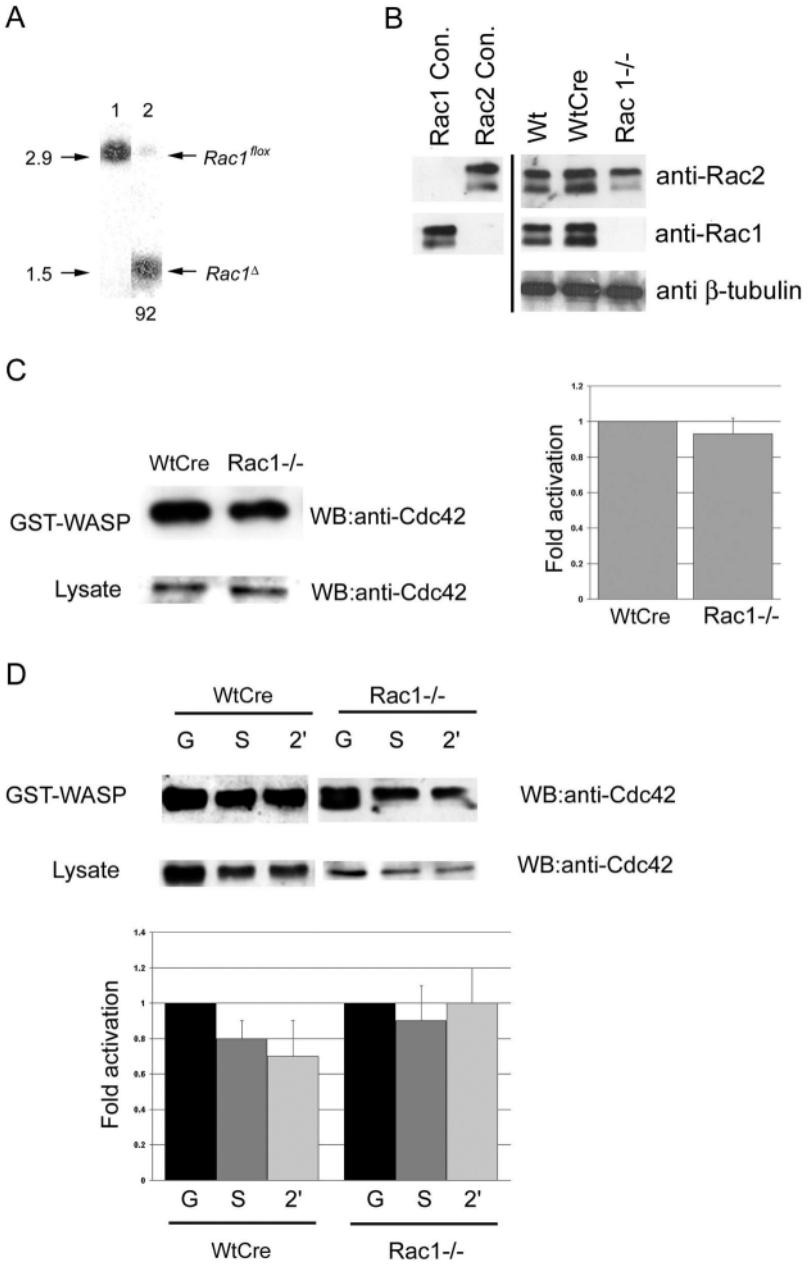

Fig. 2. Characterisation of Rac1 deletion. (A) Southern blot of DNA cut with *Bam*HI, derived from the bone marrow of a *Rac1*$^{flox/flox}$ (1) and a *Rac1*$^{flox/flox}$Mx1-Cre (2) mouse that had been injected with polyIC as described in the Materials and Methods. Bone marrow was harvested 2 days after the last polyIC injection. The Southern blot was probed with a DNA fragment from intron 3 of *Rac1* which hybridises to a 2.9 kb and a 1.5 kb fragment in the *Rac1*^{flox} and *Rac1*$^{\Delta}$ alleles respectively, as shown (Walmsley et al., 2003). The number below lane 2 shows the percentage deletion of the *Rac1*^{flox} allele. Typical deletion of the *Rac1*^{flox} allele in bone marrow was 90% or higher, and persisted for at least 3 months following polyIC injection (not shown). (B) Analysis of Rac1 and Rac2 protein expression in Wt and Rac1$^{-/-}$ BMMs. Recombinant Rac1 (Rac1 Con.) and Rac2 (Rac2 Con.) proteins (1.5 ng) purified from *E. coli* were resolved by SDS-PAGE and immunoblotted with anti-Rac2 or anti-Rac1 antibodies (left blot) to demonstrate the specificity of the antibodies. A western blot of lysates from growing Wt, WtCre and Rac1$^{-/-}$ BMMs was probed sequentially with anti-Rac2, anti-Rac1 and anti-β-tubulin antibodies (right blot). (C) Analysis of Cdc42 activity in growing cells. WtCre and Rac1$^{-/-}$ BMMs were maintained in growth medium. Cells were lysed and GST-WASP-CRIB was used to precipitate endogenous GTP-bound Cdc42. Immunoblots were performed using an anti-Cdc42-specific antibody. Immunoblots of whole cell lysates (WCL) retained from the GST-WASP-CRIB pull-down lysates were performed using the same antibody. Autoradiographs of the GST-WASP-CRIB pull-down and the WCL blots were quantified using kinetic imaging software, and the level of GTP-bound Cdc42 normalised to protein expression levels in the WCL. The results shown are the mean±s.e.m. of three independent experiments. (D) Analysis of Cdc42 activity during CSF1 stimulation. WtCre and Rac1$^{-/-}$ BMMs were maintained in growth medium (G), starved of CSF-1 overnight (S) or starved of CSF-1 overnight and re-stimulated with recombinant mCSF-1 (30 ng/ml) for 2 minutes (2′). Cells were lysed and GST-WASP-CRIB was used to precipitate endogenous GTP-bound Cdc42. Immunoblots were performed using an anti-Cdc42-specific antibody. Immunoblots of whole cell lysates (WCL) retained from the GST-WASP-CRIB pull-down lysates were performed using the same antibody. Autoradiographs of the GST-WASP-CRIB pull-down and the WCL blots were quantified using kinetic imaging software, and the level of GTP-bound Cdc42 normalised to Cdc42 expression levels in the WCL. The results shown are the mean±s.e.m. of three independent experiments.

activation in Rac1$^{-/-}$ cells because of the difficulty in detecting Rac2 expression in BMMs (Fig. 1C) and lack of sufficiently sensitive antibodies.

Rac1$^{-/-}$ BMMs have a reduced adhesive area

The morphology of growing Rac1$^{-/-}$ BMMs differed from that of the control cell populations. Rac1$^{-/-}$ BMMs were generally more elongated with small protrusive areas at both ends, whereas control cells were well spread and had large clearly identifiable lamellipodia (Fig. 3A). In addition, cultured Rac1$^{-/-}$ BMMs had significantly reduced mean adhesive area when compared to Wt and WtCre BMMs (Fig. 3B), whilst there was no significant difference between the mean adhesive areas of Wt and WtCre populations. Furthermore, Rac1$^{-/-}$ cells were also significantly more elongated than control cells (Fig. 3C). We have also observed a small but reproducible reduction in the numbers of Rac1$^{-/-}$ cells compared to control cells that adhered to tissue culture plastic (10% reduction) or fibronectin-coated dishes (15% reduction) 60 minutes after plating (data not shown).

Rac1$^{-/-}$ BMMs have a reduced morphological response to CSF-1

The BMMs generated in these experiments are dependent on

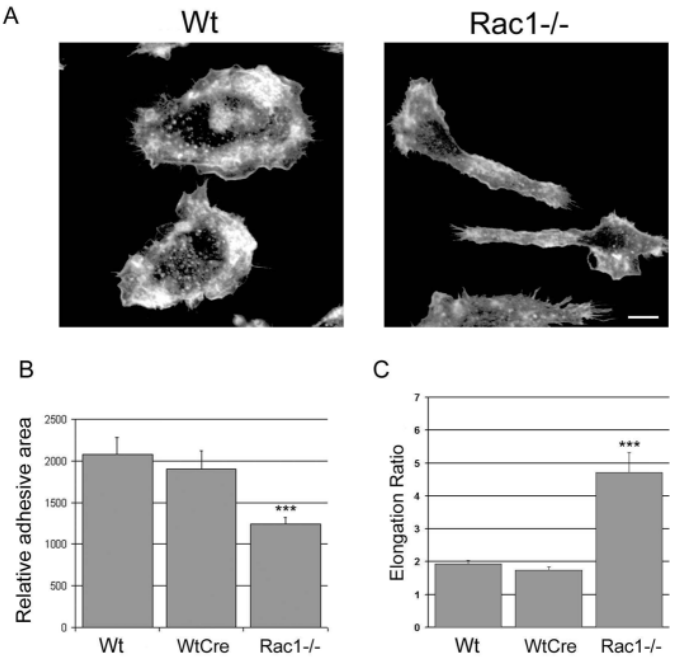

Fig. 3. Rac1$^{-/-}$ BMMs have altered morphology. (A) Growing BMMs derived from Wt and Rac1$^{-/-}$ mice were stained for F-actin with TRITC-phalloidin. Scale bar: 10 μm. (B) Quantification of adhesive area. Growing Wt, WtCre and Rac1$^{-/-}$ cells were stained for F-actin, and total area of adhesion was measured (see Materials and Methods). The results shown are mean ± s.e.m. of ≥100 cells from each population over three separate experiments. (C) Quantification of cell shape. Growing Wt, WtCre and Rac1$^{-/-}$ cells were stained for F-actin, and elongation ratio (ratio of the longest to the shortest cell axes) was measured (see Materials and Methods). The results shown are mean ± s.e.m. of ≥100 cells from each population over three separate experiments. Statistical significance compared to Wt values was calculated using Student's *t*-test, ***P<0.001.

CSF-1 for their survival. Wt BMMs when starved of CSF-1 had a significantly reduced adhesive area compared to growing cells with little or no membrane ruffling (Fig. 4A,E). This reduced adhesive area was accompanied by a significant increase in cell elongation (Fig. 4F). Stimulation with CSF-1 induced an increase in F-actin and membrane ruffling within 2-5 minutes after stimulation (Fig. 4C; arrows). In addition CSF-1 induced centrifugal spreading as indicated by the decrease in elongation ratio, and by 10 minutes the adhesive area was larger than that of unstimulated cells (Fig. 4E, F and unpublished data). Rac1$^{-/-}$ BMMs did not show a reduction in adhesive area when starved of CSF-1 (Fig. 4E) compared to growing cells. Moreover, re-stimulation with CSF-1 did not significantly affect adhesive area (Fig. 4E). The elongation ratio of CSF-1-starved Rac1$^{-/-}$ cells was lower than growing Rac1$^{-/-}$ cells (possibly because of cell contraction). However, this elongation ratio was reduced further by addition of CSF-1, indicating that Rac1$^{-/-}$ cells did respond and spread in response to CSF-1, although the total adhesive area in the presence of CSF-1 was significantly lower than control cells (Fig. 4E,F).

Role of Rac1 in CSF-1-induced membrane ruffling

Dominant negative Rac1 inhibits membrane ruffling in CSF-1-stimulated Bac1 macrophages (Allen et al., 1997). Stimulation of Rac1$^{-/-}$ cells with CSF-1 did not induce membrane ruffling to the same extent as in control cells (Fig. 4C,D), although some ruffling and actin polymerisation was still detected in protrusive regions (Fig. 4D; arrows). Furthermore some cells were able to exhibit a full ruffling response (Fig. 4D; insert). These results suggest that Rac1 is not absolutely required for CSF-1-stimulated membrane ruffling in BMMs, but that it does contribute to the process.

Residual membrane ruffling seen in Rac1$^{-/-}$ macrophages could be mediated by Cdc42. Dominant negative Cdc42 has been reported to inhibit phagocytosis and membrane ruffling in the RAW macrophage cell line (Cox et al., 1997), although in other macrophage cell lines it primarily reduces filopodium formation (Allen et al., 1997; Peppelenbosch et al., 1999). However, BMMs do not extend filopodia when stimulated with CSF-1 (Fig. 4D). Expression of V12Cdc42 in BMMs induced the formation of filopodia and some membrane ruffling, although far more extensive ruffling was induced by expression of V12Rac1 (data not shown). To determine whether Cdc42 contributed to CSF-1-induced ruffling in Rac1$^{-/-}$ BMMs we expressed N17Cdc42 in CSF-1-starved Rac1$^{-/-}$ and control cells. Cells were then stimulated with CSF-1 for 5 minutes and scored for membrane ruffles. Expression of N17Cdc42 induced cell rounding in a proportion of the BMM population: 51±3% of Rac1$^{-/-}$ cells were rounded compared to 36±9% of the WtCre BMMs. This suggests that Rac1$^{-/-}$ cells are less able to compensate for the effects of expressing N17Cdc42, and that Cdc42 and/or a related GTPase inhibited by N17Cdc42, contributes to BMM cell spreading and/or contractility.

Expression of N17Cdc42 in Rac1$^{-/-}$ cells reduced the percentage of cells with a normal ruffling response to just 27% (Fig. 5A), and completely ablated the ruffling response in some Rac1$^{-/-}$ cells (Fig. 5B). Rounded cells were excluded from the ruffling assay, as it was not possible to observe ruffling on rounded cells. Expression of N17Cdc42 also reduced the percentage of cells with a normal ruffling response to 68% and 52% in Wt and WtCre cells respectively (Fig. 5A). However, N17Cdc42 did not completely inhibit ruffling, suggesting that another signalling pathway is responsible for a proportion of the CSF-1-induced ruffling in Rac1$^{-/-}$ cells.

Interestingly, expression of N17Rac1 reduced the Wt BMM ruffling response to a level similar to that of Rac1$^{-/-}$ BMMs, thus expression of N17Rac1 was unable to abolish the ruffling response of Wt cells completely (Fig. 5A). These results together with our observation that Rac1-deficient cells can still generate membrane ruffles indicate that other signalling pathways in addition to Rac1 contribute to membrane ruffling.

Exogenous expression of Rac1 rescues the Rac1$^{-/-}$ phenotype

To establish whether the reduction in adhesive area and ruffling response in the Rac1$^{-/-}$ macrophages could be rescued by exogenous expression of Rac1 we expressed WtRac1 in Rac1$^{-/-}$ BMMs. Expression of WtRac1 restored the ruffling response to CSF-1 to control levels (Fig. 5D, arrows) and significantly decreased the elongation ratio of CSF-1-stimulated Rac1$^{-/-}$ cells, with a concomitant increase in adhesive area (Fig. 5F and unpublished data). Conversely,

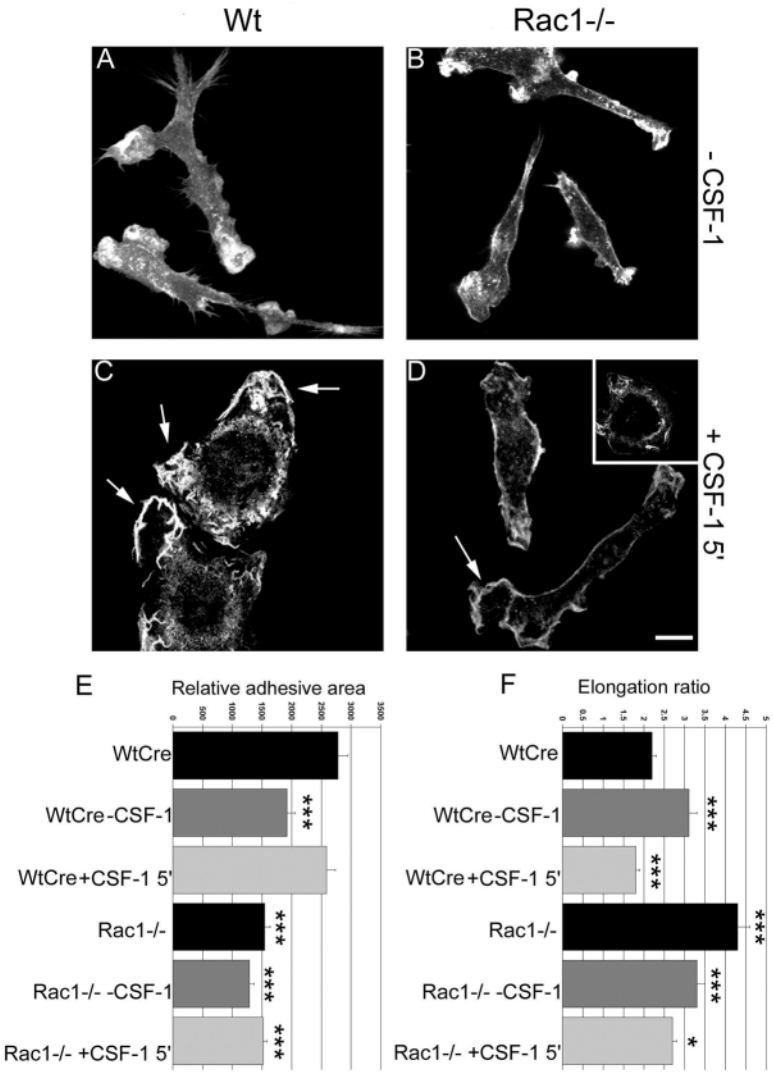

Fig. 4. Rac1$^{-/-}$ BMMs have a reduced ruffling response to CSF-1. CSF-1-starved (A,B) and CSF-1-stimulated BMMs (C,D) were fixed and stained for F-actin with TRITC-phalloidin. Arrows indicate membrane ruffles. (Inset) An example of a Rac1$^{-/-}$ BMM that has a full ruffling response to CSF-1. Scale bar: 10 μm. (E,F) Quantification of changes in adhesive area (E) and cell elongation ratio (ratio of the longest to the shortest cell axes; F) in growing, starved and CSF-1 stimulated WtCre and Rac1$^{-/-}$ BMMs. The results shown are mean±s.e.m. of ≥100 cells from each population over two separate experiments. Statistical significance compared to WtCre growing values was calculated using Student's *t*-test, **P*<0.05; ****P*<0.001.

exogenous expression of N17Rac1 in WtCre cells significantly increased the elongation ratio of these cells with a concomitant reduction in adhesive area (Fig. 5F and unpublished data).

Rac1$^{-/-}$ BMMs migrate normally in response to CSF-1

Rac proteins are generally considered to be essential for cell migration (Etienne-Manneville and Hall, 2002; Ridley, 2001). To assess whether Rac1 was specifically required for macrophage migration, we initially investigated the random migration of Rac1$^{-/-}$ and control BMMs on glass and plastic. Migration speed was unaltered in the Rac1$^{-/-}$ population compared to control cells (Table 1; see also movies 1 and 2, http://jsc.biologists.org/supplemental/). Macrophages exhibit positive chemotaxis towards CSF-1 (Allen et al., 1998; Webb et al., 1996). Similarly, Rac1$^{-/-}$ cells showed chemotaxis towards CSF-1 and their speed of migration was not significantly different from control populations (Table 1). From studying the timelapse microscopy of migrating Rac1$^{-/-}$ cells (Movie 2, http://jsc.biologists.org/supplemental/) we observed that although these cells were more elongated than control cells they were still able to generate membrane protrusions in the direction of migration. It was not possible to determine the effect of dominant negative Cdc42 on the migration of BMMs because of the low expression efficiency of plasmids microinjected or transfected in these cells. However, our results clearly demonstrate that Rac1 is not essential for migration.

Discussion

We have characterised for the first time the morphology and motile behaviour of Rac1$^{-/-}$ cells. Our results demonstrate that Rac1 contributes to normal cell spreading and CSF-1-induced membrane ruffling in macrophages. Surprisingly however, loss of Rac1 does not affect the migration speed or chemotaxis of macrophages.

Rac1 is generally considered to be essential for normal migration based on results using constitutively active (Rac1V12/L61) or dominant negative proteins (e.g. N17Rac1). N17Rac mutants are believed to inhibit Rac activity by sequestering exchange factors, which may be required by all of the Rac isoforms (Feig, 1999). There is, to date, no evidence that N17Rac1 specifically inhibits Rac1 activity in any cell type. In macrophages N17Rac1 may sequester exchange factors required by Rac2, which is known to be required for migration in other haematopoietic cell lineages (Croker et al., 2002a; Yang et al., 2001). Indeed, we have observed that N17Rac1 induces morphological changes in both

Table 1. Mean speed of migration

Genotype	Substrate	Mean speed μm/min	+ve chemotaxis to CSF-1	*n*
WtCre	Glass	0.36±0.006	na	55
Rac1$^{-/-}$	Glass	0.31±0.004	na	70
WtCre	Plastic	0.6±0.004	na	158
Rac1$^{-/-}$	Plastic	0.6±0.004	na	143
Wt	Glass (Dunn)	0.3±0.003	Yes	64
WtCre	Glass (Dunn)	0.35±0.003	Yes	112
Rac1$^{-/-}$	Glass (Dunn)	0.3±0.003	Yes	67

To analyse random migration, cells were seeded at a density of 2×10^4/ml on either glass coverslips or plastic dishes and filmed for 16 hours. Results shown here are the mean of at least three independent experiments. For Dunn chamber analysis, cells were seeded at 2×10^4/ml, starved of CSF-1 for 8 hours and then filmed in a Dunn chamber (see Materials and Methods) in a gradient of CSF-1 for 20 hours. Results shown here are the mean of at least three independent experiments ±SD. na, not applicable.

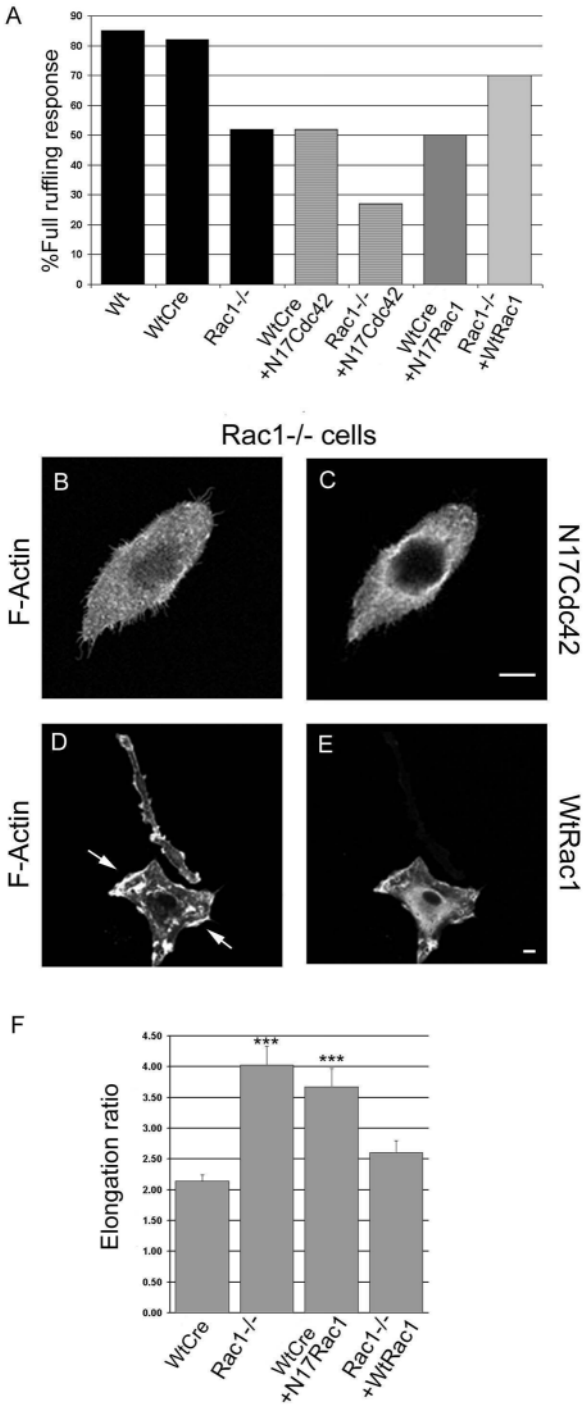

Fig. 5. Effects of N17Cdc42, N17Rac1 and WtRac1 on BMMs. **(**A) Quantification of the ruffling response to CSF-1. CSF-1-starved control (Wt or WtCre) or Rac1$^{-/-}$ cells were microinjected with 100 ng/μl Flag-tagged N17Cdc42, N17Rac1 or WtRac1 cDNA, as indicated below each bar. The cells were incubated for 3 hours prior to re-stimulation with 30 ng/ml CSF-1 for 5 minutes. Following stimulation, the cells were fixed and stained for F-actin. The ruffling response to CSF-1 was scored as described in Materials and Methods. Black bars, uninjected control and Rac1$^{-/-}$ cells. (B-E) Ruffling response in microinjected Rac1$^{-/-}$ cells. Cells were microinjected with 100 ng/μl Flag-tagged N17Cdc42 or WtRac1 cDNA. The cells were incubated for 3 hours prior to re-stimulation with 30 ng/ml CSF-1 for 5 minutes. Following stimulation, the cells were fixed and stained for F-actin. Arrows indicate membrane ruffling. Scale bar: 10 μm. (F) Quantification of cell elongation ratio following exogenous expression of N17Rac1 and WtRac1 as described above. The elongation ratio (ratio of the longest to the shortest cell axes) was then measured (see Materials and Methods). The results shown are mean±s.e.m. of 60 cells from each population over at least three separate experiments. Statistical significance compared to WtCre values was calculated using Student's *t*-test, ***P<0.001.

Wt and Rac1$^{-/-}$ BMMs, indicating that it does not specifically inhibit Rac1 (our unpublished data).

We have shown that BMMs only express Rac1 and Rac2, and unlike other cells of haematopoietic origin Rac1 rather than Rac2 is the predominant isoform in BMMs. It is clear from our results that Rac1 and Rac2 are not functionally redundant in BMMs, as loss of Rac1 alone is sufficient to affect cell morphology and membrane ruffling. However, the loss of Rac1 does not affect the ability of BMMs to migrate at normal speeds, and this could be due to compensation by Rac2 or another related GTPase or it may be that in BMMs Rac2 is primarily responsible for cell migration. Indeed a 3-fold increase in Rac1 activity in Rac2$^{-/-}$ neutrophils was unable to compensate for a severe migratory phenotype (Li et al., 2002). These results along with those of others on Rac2$^{-/-}$ cells (Croker et al., 2002a; Croker et al., 2002b; Roberts et al., 1999; Yang et al., 2001) suggest that the Rac proteins have specific as well as overlapping functions and so there must be mechanisms within a single cell to achieve this isoform specificity.

Although Rac1, Rac2 and Rac3 proteins are highly homologous there is some sequence divergence in the carboxy-terminal region, in the polybasic domain adjacent to the prenylation site. In Rac1 the polybasic domain is composed of six consecutive basic residues, whereas in Rac2, three of the residues are replaced with neutral amino acids. It is now known that at least in Rac2 this polybasic region is essential for biological function and intracellular localisation (Tao et al., 2002). Thus sequence divergence in the polybasic region may generate isoform specificity through differential localisation of Rac1/2/3 within the cell membrane domains. Specificity could also be achieved by selective interaction between each Rac isoform and a subset of GEFs and GAPs. For example, Rac1 could preferentially interact with an integrin-activated GEF and subsequently activate a subset of Rac effectors to affect cell morphology.

The Rac pathway is activated during integrin-mediated cell adhesion, and dominant-negative Rac1 inhibits cell spreading on integrin ligands (Berrier et al., 2000; Mettouchi et al., 2001; Ory et al., 2000; Price et al., 1998). Integrin activation of Rac has also been reported to contribute to induction of cyclin D1 expression and cell cycle progression in endothelial cells (Mettouchi et al., 2001), but we did not observe a difference in cyclin D1 expression or cell proliferation in Rac1$^{-/-}$ cells (data not shown). Rac may induce cell spreading by regulating the phosphorylation of myosin II heavy chain and thus reducing cell contractility (van Leeuwen et al., 1999) and/or by stimulating actin polymerisation through activation of WAVE proteins and the Arp2/3 complex (Demerol et al., 2002).

Rac1$^{-/-}$ cells have reduced membrane ruffling in response to CSF-1, but there is no detectable effect on CSF-1-induced migratory behaviour, suggesting an uncoupling of migration and membrane ruffling. Consistent with this hypothesis, detailed analysis of the forces produced during macrophage migration led to the conclusion that extension of the leading lamella correlated well with force production but that generalised ruffling did not (Guilford et al., 1995). In addition inhibition of MAPK activity in response to EGF blocked cell migration without impacting on the ability of Rac to promote membrane ruffling (Leng et al., 1999). Indeed, Leng et al. demonstrated that whilst MAPK activity is required for cell migration it has no effect on membrane ruffling. In BMMs is it possible that membrane ruffling is more important for pinocytosis and/or phagocytosis than for migration. Dominant-negative Rac1 inhibits macrophage phagocytosis (Castellano et al., 2001; Leverrier and Ridley, 2001) and we therefore aim to investigate the phagocytic potential of Rac1$^{-/-}$ cells. We have shown that dominant negative Cdc42 reduces membrane ruffling in control and Rac1$^{-/-}$ cells, suggesting that in macrophages Cdc42, or a closely related protein, also regulates the formation of membrane ruffles in addition to Rac1. This could either be upstream of Rac2 or on a separate signalling pathway.

Although generally Rac1 is thought to be an important regulator of cell migration, expression of N17Rac1 in colon carcinoma cells and rat 1 fibroblasts did not inhibit cell migration (Ahram et al., 2000; O'Connor et al., 2000). Rac activation can also inhibit migration by downregulating Rho activity (Sander et al., 1999), and thus the relative contribution of Rac to migration depends on cell type and conditions. Cell migration speed is thought to be biphasically dependent on cell-substratum adhesion whereby the maximum speed attainable varies depending on ligand concentration, integrin expression or integrin-ligand affinity (Palecek et al., 1997). Thus Rac1$^{-/-}$ macrophages are presumably able to achieve normal migration speeds because the level of cell-substratum adhesion is similar to Wt macrophages. Recent evidence suggests that migration speed is also dependent on the rate of effective protrusion at the leading edge (Bear et al., 2000; Bear et al., 2002; Pollard and Borisy, 2003). Rac1$^{-/-}$ macrophages can still generate membrane protrusions in response to CSF and this level of protrusive activity is presumably sufficient to maintain normal migration speeds.

In summary, we have demonstrated that Rac1 is not required for detection of a chemokine gradient, nor is it required for cell migration or small actin-based protrusions in primary bone marrow-derived macrophages. However Rac1 is required for cell spreading and the generation of membrane ruffles in response to CSF-1.

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