A WAVE2-Abi1 complex mediates CSF-1-induced F-actin-rich membrane protrusions and migration in macrophages

Wassim Abou Kheir[1,*], Jean-Claude Gevrey[1,*], Hideki Yamaguchi[1], Beth Isaac[1] and Dianne Cox[1,2,‡]

[1]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York City, NY 10461, USA
[2]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York City, NY 10461, USA
*These authors contributed equally to this work
‡Author for correspondence (e-mail: dcox@aecom.yu.edu)

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Summary

Colony-stimulating factor 1 (CSF-1) is an important physiological chemoattractant for macrophages. The mechanisms by which CSF-1 elicits the formation of filamentous actin (F-actin)-rich membrane protrusions and induces macrophage migration are not fully understood. In particular, very little is known regarding the contribution of the different members of the Wiskott-Aldrich Syndrome protein (WASP) family of actin regulators in response to CSF-1. Although a role for WASP itself in macrophage chemotaxis has been previously identified, no data was available regarding the function of WASP family verprolin-homologous (WAVE) proteins in this cell type. We found that WAVE2 was the predominant isoform to be expressed in primary macrophages and in cells derived from the murine monocyte/macrophage RAW264.7 cell line (RAW/LR5). CSF-1 treatment of macrophages resulted in WAVE2 accumulation in F-actin-rich protrusions induced by CSF-1. Inhibition of WAVE2 function by expressing a dominant-negative mutant or introducing anti-WAVE2 antibodies in RAW/LR5 cells, as well as reduction of endogenous WAVE2 expression by RNA-mediated interference (RNAi), resulted in a significant reduction of CSF-1-elicited F-actin protrusions. WAVE2 was found in a protein complex together with Abelson kinase interactor 1 (Abi1) in resting or stimulated cells. Both WAVE2 and Abi1 were recruited to and necessary for the formation of F-actin protrusions in response to CSF-1. Reducing the levels of WAVE2, directly or by targeting Abi1, resulted in an impaired cell migration to CSF-1. Altogether these data identify a WAVE2-Abi1 complex crucial for the normal actin cytoskeleton reorganization and migration of macrophages in response to CSF-1.

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Key words: WAVE2, Abi1, Macrophage, Actin cytoskeleton, Cell migration

Introduction

Macrophages, which are scattered throughout every tissue, represent a key component of the immune system through their ability to phagocytose foreign material and dying cells, release cytokines and function as antigen-presenting cells. Recruitment of macrophages (or monocytes, their immediate precursors) at specific sites is therefore an important process in achieving a localized and efficient response, and is dependent on their ability to respond chemotactically to a variety of secreted molecules. The pleiotropic colony-stimulating factor-1 (CSF-1, also known as macrophage CSF) is a major chemotactic factor for macrophages as well as a primary regulator of their differentiation, survival and proliferation (reviewed by Pixley and Stanley, 2004). Enhanced production of CSF-1 and the associated macrophage recruitment have also been shown to promote the progression of diseases such as rheumatoid arthritis (Bischof et al., 2000; Campbell et al., 2000), atherosclerosis (Rajavashisth et al., 1998; Smith et al., 1995) and breast cancer (Aharinejad et al., 2004; Lin et al., 2001), implying that the effect of CSF-1 on macrophages has both physiological and clinical relevance.

CSF-1 treatment of macrophages leads to a rapid reorganization of the actin cytoskeleton leading to profound cell morphological changes such as extension of membrane protrusions. These early events are required for and followed by the directed migration of macrophages toward the source of CSF-1 (Allen et al., 1997; Boocock et al., 1989; Webb et al., 1996). All CSF-1 effects are exclusively mediated by the cell surface receptor tyrosine kinase CSF-1R. Upon ligand binding, CSF-1R undergoes activation through autophosphorylation of tyrosine residues and the CSF-1 signal is then transduced through a number of downstream molecules that associate, directly or not, with the receptor (Pixley and Stanley, 2004). While the cell signaling events important for macrophage chemotaxis to CSF-1 remain incompletely elucidated, several molecules have been identified as critical components in various steps of this process. These include the p110 catalytic subunit isoforms β and γ of class IA phosphatidylinositide 3-kinases (PI 3-kinase) (Vanhaesebroeck et al., 1999) and the

small Rho GTPases Rho, Rac and Cdc42 (Allen et al., 1997; Allen et al., 1998; Cox et al., 1997). How these proteins are connected to regulators of the actin cytoskeleton and support cell migration in macrophages is ill-defined.

In different cell types, the Rho GTPase-dependent assembly of filamentous actin (F-actin) has been shown to be mediated by members of the Wiskott-Aldrich Syndrome protein (WASP) family of proteins (reviewed by Millard et al., 2004; Stradal et al., 2004; Takenawa and Miki, 2001). In mammalian cells, this family comprises five members: WASP, the closely related neural WASP (N-WASP) and WASP family verprolin-homologous (WAVE) proteins WAVE1, 2 and 3. The common feature of these proteins is the presence at their C-terminal end of a verprolin homology cofilin homology acidic (VCA) module, which is necessary and sufficient to interact with the Arp2/3 complex and to stimulate actin nucleation and polymerization (Millard et al., 2004; Stradal et al., 2004; Takenawa and Miki, 2001). Adjacent to the VCA domain, a proline-rich region acts as a binding site for SH3 domain-containing proteins and is also shared by all WASP family proteins. The N-termini are more divergent, with WASP and N-WASP bearing a WASP homology domain (WH1), followed by a short basic region and a GTPase-binding domain (GBD) capable of binding GTP-loaded Cdc42, whereas WAVE proteins possess their own N-terminal WAVE homology domain (WHD) immediately followed by a basic stretch and, importantly, they have no domain comparable to the GBD, indicating distinct modes of regulation of WAVEs compared to WASP and N-WASP (Millard et al., 2004; Stradal et al., 2004; Takenawa and Miki, 2001).

So far, among the WASP/WAVE proteins, only WASP has been shown to be important for directionality of cell movement toward CSF-1 without apparently being required for CSF-1-stimulated motility per se in macrophages (Jones et al., 2002; Zicha et al., 1998). This leads to the proposal that, besides WASP itself, other members of the WASP family are likely to mediate, at least in part, the cytoskeletal reorganization and the associated motility/chemotaxis observed in macrophages in response to CSF-1. Given the extremely low level of expression of N-WASP in macrophages (Suzuki et al., 2002), WAVE proteins appear to be the most obvious candidates to play such roles. The aim of this investigation was therefore to determine the expression pattern of the different WAVE proteins in macrophages and to study their involvement in CSF-1-induced actin cytoskeleton remodeling and cell migration. Herein, we provide evidence that WAVE2 is the WAVE isoform that is predominantly expressed in primary macrophages and in the RAW/LR5 monocyte/macrophage cell line and we describe a functional role for a WAVE2/Abelson kinase interactor 1 (Abi1) complex in the formation of F-actin rich protrusions and cell migration in response to CSF-1.

Materials and Methods

Cells and reagents

RAW/LR5 cells were derived from the murine monocyte/macrophage RAW 264.7 cell line (Cox et al., 1997) and were grown in RPMI medium (Mediatech Inc., Herndon, VA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma, St Louis, MO, USA). Murine bone marrow-derived macrophages (BMM) were isolated as described previously (Stanley, 1997) and were grown in α-MEM containing 15% FBS, 360 ng/ml recombinant human CSF-1 (Chiron, Emeryville, CA) and antibiotics. All cells were maintained at 37°C in an incubator with 5% CO_2. Rabbit polyclonal anti-WAVE1 and anti-WAVE2 antibodies (Yamazaki et al., 2003) were a gift from Tadaomi Takenawa (University of Tokyo, Japan). Rabbit polyclonal anti-WASP, goat polyclonal anti-WAVE2 and anti-Abi1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-Abi1 was from MBL (Woburn, MA, USA). Mouse monoclonal anti-β-actin and anti-FLAG were from Sigma. Mouse monoclonal anti-Myc was from Cell Signaling Technology (Beverly, MA, USA). The horseradish peroxidase (HRP)-conjugated antibodies against rabbit, mouse or goat IgG, the biotin-conjugated and Cy3-conjugated donkey anti-mouse IgG were from Jackson ImmunoResearch (West Grove, PA, USA). All secondary antibodies conjugated to Alexa Fluor 488 or 568 as well as Alexa Fluor 568 phalloidin and Alexa Fluor 568 streptavidin were from Molecular Probes (Eugene, OR, USA). Protein A/G plus-agarose beads were from Santa Cruz. The SuperFect transfection reagent was from Qiagen (Valencia, CA, USA). Murine CSF-1 was from R&D Systems (Minneapolis, MN, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was isolated from cells or tissues using TRIzol reagent from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR was performed using the one step RT-PCR kit from Qiagen. Sets of primers specific for each mouse WAVE isoform were as follows: WAVE1 forward 5′-CCACCCTGCCTGTAATC-AGT-3′, reverse 5′-GATAGTGGCCACGTCGTTTT-3′; WAVE2 forward 5′-AGCTGACTACCCAATGCCAC-3′, reverse 5′-TCA-CAGCAGGCAATGAAGAC-3′; WAVE3 forward 5′-ACCCGAGA-GGTGAAAAAGGT-3′, reverse 5′-CCAGCTGTGTAGGAGGGT-GT-3′.

Constructs and cell transfection

FLAG-tagged WAVE1, FLAG-tagged WAVE2, FLAG-tagged WAVE1ΔV, FLAG-tagged WAVE2ΔV and Myc-tagged WASPΔC, all subcloned in pEF-BOS vector (Miki et al., 1998; Suetsugu et al., 1999), as well as the human GFP-WAVE2 plasmid, were a gift from Tadaomi Takenawa (University of Tokyo, Japan). pEF-BOS/FLAG-WASP was a gift from Donn M. Stewart (National Cancer Institute, USA). Transient transfections were performed using the SuperFect reagent from Qiagen according to the manufacturer's instructions.

Cell stimulation

For all experiments involving a CSF-1 stimulation (with the exception of the cell migration assay), cells were treated as follows: adherent RAW/LR5 cells were serum starved for at least 1 hour at 37°C, followed by equilibration for 10 minutes in BWD buffer (20 mM Hepes, 125 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 5 mM glucose, 10 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM $CaCl_2$; pH 7.4). Some cells were then stimulated with 20 ng/ml CSF-1 in BWD for 5 minutes at 37°C and the controls were left untreated. The stimulation was promptly stopped by fixing the cells with formaldehyde (for immunofluorescence experiments) or by cell lysis in ice-cold buffer (for western blotting and immunoprecipitation). BMM were CSF-1-deprived overnight to upregulate CSF-1 receptor expression, prior to equilibration in BWD and treatment identical to that described for RAW/LR5 cells.

Immunoprecipitation and western blotting

Cells were lysed in ice cold lysis buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF; pH 7.4). Whole cell lysates were

then clarified by centrifugation at 16,000 ***g*** for 10 minutes at 4°C and either used for immunoprecipitation (IP) (see below) or mixed with 5× Laemmli buffer and boiled for 5 minutes. IPs were carried out by a first incubation of cell lysates at 4°C with control IgG prebound to protein A/G agarose beads (preclear step) followed by centrifugation and incubation of the precleared lysates at 4°C with specific antibody prebound to beads. Beads were then pelleted, washed with lysis buffer and finally resuspended in Laemmli buffer and boiled for 5 minutes. Samples were resolved by SDS-PAGE and proteins were transferred onto PVDF membranes (Immobilon-P, Millipore). Membranes were then blocked with either 5% skimmed milk (when rabbit or mouse antibodies were used) or 5% donkey serum (when goat antibodies were used) in TBS containing 0.1% Tween 20 (TBS-T) followed by an overnight incubation with primary antibodies at 4°C. Membranes were then washed with TBS-T and incubated with secondary antibodies conjugated to HRP. Signals were detected using the SuperSignal West Pico Chemiluminescent Substrate from Pierce (Rockford, IL) and images were acquired using a Kodak Image Station 440.

Immunofluorescence microscopy

Following stimulation, cells plated on 12 mm glass coverslips were fixed in 3.7% formaldehyde (in BWD) for 7 minutes, then permeabilized in 0.2% Triton X-100 (in BWD) for 5 minutes, and blocked with 1% BSA. F-actin was visualized by staining with Alexa Fluor 568 phalloidin. WAVE2 was detected using goat anti-WAVE2 antibody and Alexa Fluor 488 donkey anti-goat IgG. Expression of epitope-tagged WAVE/WASP proteins was detected using anti-FLAG or anti-Myc antibodies and Alexa Fluor 488 goat anti-mouse IgG. Abi1 was detected using monoclonal mouse anti-Abi1 antibody and either biotin-anti-mouse IgG followed by Alexa Fluor 568 streptavidin (BMM in Fig. 4C) or donkey anti-mouse IgG conjugated to Cy3 (RAW/LR5 in Fig. 4C). All images were taken with a confocal laser-scanning microscope (model radiance 2000, Bio-Rad Laboratories, Hercules, CA) except for the image in Fig. 5A, taken with an Olympus microscope equipped with a cooled CCD camera. Images were processed with Adobe Photoshop.

Quantification of F-actin rich membrane protrusions

Using fluorescence microscopy, a protrusion was defined by the presence of F-actin-rich submembranous folds. The extent of CSF-1-induced F-actin-rich membrane protrusions was scored using a scale of 0-3 (modified from Cox et al., 1997), where 0=no protrusion, 1=protrusions in one area of the cell, 2=protrusions in two distinct areas of the cell, 3=protrusions in more than two distinct areas of the cell. The protrusion index was calculated as the average of protrusion scores of at least 60 cells and was expressed as a percentage of control. For cells expressing FLAG- and Myc-tagged proteins, at least 60 cells positive for the epitope were analyzed and the extent of protrusions was quantified and expressed as a percentage of non transfected cells on the same coverslip.

Phagocytosis assay

Cells plated on 12 mm glass coverslips were transiently transfected with the indicated constructs and the ability of cells to perform Fcγ receptor-mediated phagocytosis was assessed as described previously (Cox et al., 1997). Briefly, cells were incubated with IgG-coated erythrocytes for 30 minutes at 37°C in BWD. Non internalized particles were then removed by washing with BWD followed by hypotonic lysis. Cells were then fixed and stained for the indicated constructs and the average number of ingested particles was calculated in at least 100 cells for each condition and expressed as a percentage of the number of ingested particles in non transfected cells on the same coverslip.

Cytosolic loading

Anti-WAVE2 antibodies were introduced into the cytosol of adherent RAW/LR5 cells as described previously (Cox et al., 2002). In brief, cells were transiently permeabilized with glass beads in the presence of rhodamine-dextran and either 0.4 mg/ml goat anti-WAVE2 or control goat IgG. After 1 hour recovery, cells were stimulated with CSF-1, fixed and stained for F-actin as described above. The extent of CSF-1-induced protrusions (using the scoring method described above) was determined for 30 rhodamine-positive antibody-loaded cells and expressed as a percentage of rhodamine-negative cells on the same coverslip.

RNA-mediated interference (RNAi)

Reduction of endogenous WAVE2 and Abi1 expression in RAW/LR5 cells was performed using the pSUPER RNAi system (Oligoengine, Seattle, WA, USA) according to the manufacturer's instructions. Two distinct oligonucleotides within the open reading frame of mouse WAVE2 (W2sh#1: 176-194) and (W2sh#2: 234-252) or mouse Abi1 (Abish#1: 169-187) and (Abish#2: 197-215), were used as target sequences. Inserts coding for short hairpin RNA (shRNA) against WAVE2 or Abi1 transcript were cloned between the *Bgl*II and *Hin*dIII restriction sites of the pSUPER.retro.puro vector. The resulting plasmids were transfected into a 293T-based packaging cell line and the cell culture supernatants were used to retrovirally infect RAW/LR5 cells. Stable mass population and single clones with reduced WAVE2 and Abi1 expression were obtained after puromycin selection (resistance brought by the pSUPER.retro.puro plasmid) of the infected cells. Control cells were generated by retroviral infection of a pSUPER plasmid coding for a mock shRNA sequence. The levels of WAVE2 or Abi1 protein expression were monitored by western blotting.

Cell migration assay

Cell migration was measured using a transmigration chamber assay with 8 μm pore size inserts (Falcon), according to the manufacturer's instructions. Briefly, the inserts were placed into 24-well plates containing RPMI in the presence or absence of 20 ng/ml CSF-1. 500,000 serum-starved cells were then loaded onto the inserts and incubated at 37°C for 4 hours. Cells that had migrated through the inserts were counted using phase microscopy and the average number of cells in 15-20 different fields was calculated. Cell migration in response to CSF-1 was expressed as fold induction compared to the corresponding condition in the absence of CSF-1. CSF-1-induced migration of control cells (mock-shRNA-treated) was set to 100 and the relative ability of WAVE2 or Abi1 shRNA-treated cells to respond to CSF-1 was represented as a percentage of mock-treated cells.

Data analysis

Significance of the data was analyzed using Student's *t*-test, and differences between two means with a P value <0.05 were considered significant. Error bars represent the standard error of the mean.

Results

Expression of WAVEs in macrophages

The relative expression of the three WAVE isoforms has not been determined in macrophages. The presence or absence of WAVE transcripts in the murine monocyte/macrophage RAW/LR5 cell line and in primary murine thioglycollate-elicited peritoneal macrophages was therefore examined by RT-PCR. Using sets of primers specific for each isoform, amplicons corresponding to mRNA for WAVE1 and WAVE2 but not for WAVE3 were detected in both samples (Fig. 1A).

WAVE3 transcript was detected in RNA isolated from mouse brain used as a positive control for WAVE3 expression. No signal was detected when omitting the reverse transcription step ruling out the possibility of a genomic DNA contamination in the preparation of samples. To determine whether the detected transcripts were indicative of protein expression, WAVE1 and WAVE2 expression was evaluated by western blotting and compared with WASP expression (Fig. 1B,C). The relative amount of WASP, WAVE1 and WAVE2 proteins was determined using specific antibodies, and signal intensities were directly compared to standards represented by samples from COS-7 cells transfected with FLAG-tagged WASP, WAVE1 and WAVE2 constructs. The relative expression levels of standards were also directly comparable using FLAG detection, making possible the determination of the WASP vs WAVE1 vs WAVE2 protein ratio in macrophages. An extremely low level of WAVE1 was barely detectable in RAW/LR5 cells and was virtually absent in BMMs. By contrast, WAVE2 was significantly expressed at levels comparable to WASP in both RAW/LR5 cells and BMM (Fig. 1B,C). A similar pattern of WAVE isoform expression was

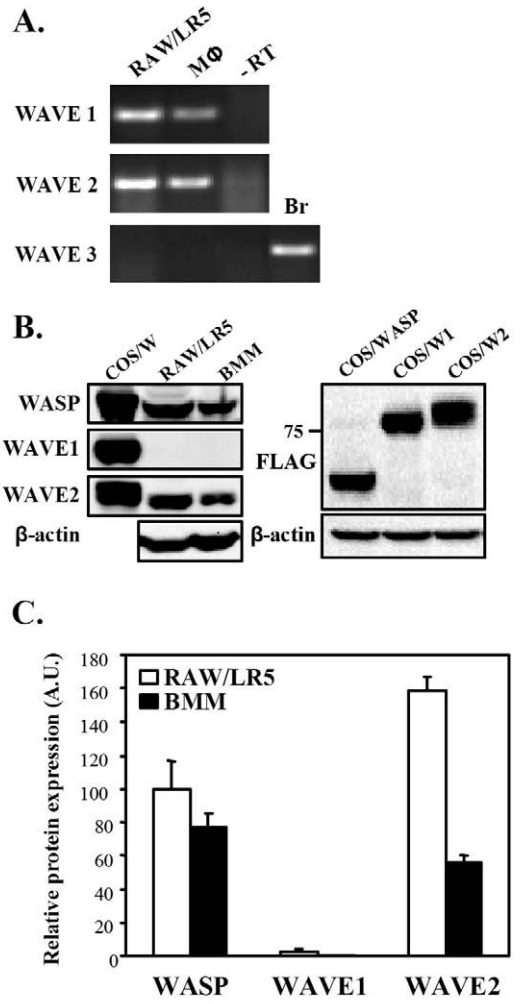

Fig. 1. Expression of WAVE family members in macrophages. (A) Total RNA was isolated from equivalent numbers of cells of the murine monocyte/macrophage RAW/LR5 cell line and primary murine thioglycollate-elicited peritoneal macrophages (MΦ) and RT-PCR was performed using sets of primers specific for the indicated transcripts. As a negative control the reverse transcription step was omitted (–RT). RNA from brain (Br) was used as a positive control for WAVE3 expression. (B) Western blot analysis of WASP, WAVE1 and WAVE2 protein expression in RAW/LR5 cells and bone marrow-derived macrophages (BMM) using isoform-specific rabbit polyclonal antibodies. Lysates from COS-7 cells transfected with either FLAG-tagged WASP (COS/WASP), FLAG-tagged WAVE1 (COS/W) or FLAG-tagged WAVE2 (COS/W2) were used as standards and probed with the indicated antibodies. (C) Corresponding quantitative analysis: WASP/WAVE over β-actin signals in macrophage samples were quantified and expressed as a percentage of their corresponding standard (COS/W with W standing for WASP or WAVE1 or WAVE2). WASP/WAVE normalized intensities were subsequently compared between each other using the relative expression of standards determined by means of FLAG detection. Numbers were finally expressed as percentages of WASP expression in RAW/LR5 cells. *n*=3 different determinations on different lysates.

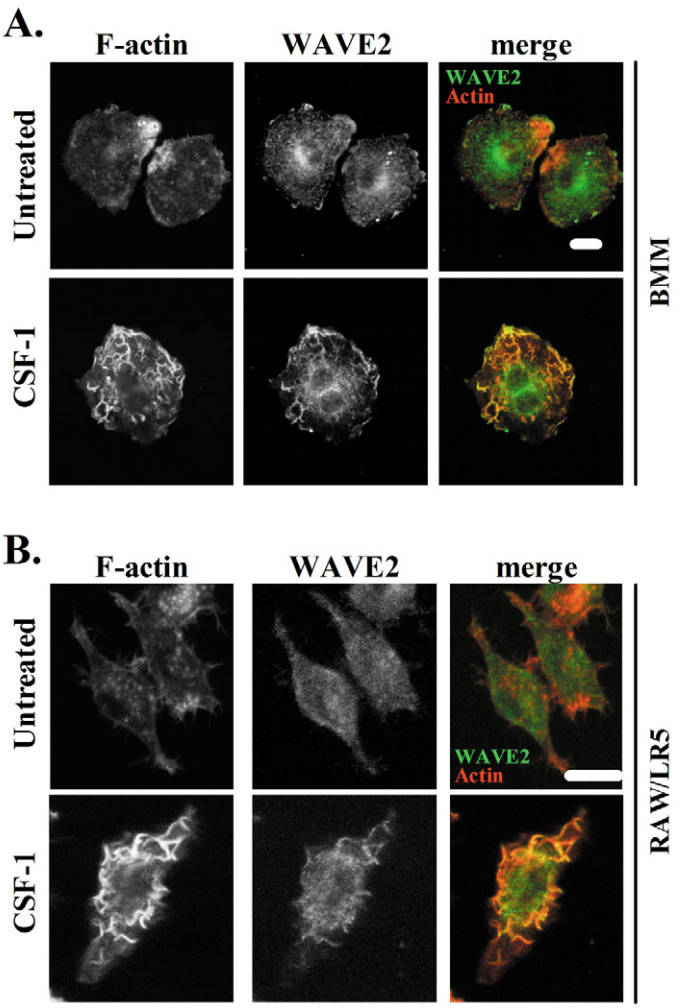

Fig. 2. WAVE2 is enriched in F-actin rich membrane protrusions elicited by CSF-1 in macrophages. BMM (A) or RAW/LR5 cells (B) were left untreated or were treated with 20 ng/ml CSF-1 for 5 minutes as described in Materials and Methods. Cells were then fixed and stained for F-actin using Alexa Fluor 568 phalloidin, and for WAVE2, using a polyclonal goat antibody followed by Alexa Fluor 488 anti-goat IgG. Bars, 10 μm.

observed in human monocyte-derived macrophages (data not shown).

WAVE2 appeared to be the only WAVE protein to be expressed at a significant level in macrophages and we therefore sought to examine its function with respect to actin reorganization in response to CSF-1.

Requirement of WAVE2 in CSF-1-induced F-actin-rich membrane protrusions

The subcellular localization of WAVE2 in macrophages in the presence or absence of CSF-1 was examined by indirect immunofluorescence confocal microscopy (Fig. 2). WAVE2 was mainly found in the cytosol of resting cells and occasionally in the perinuclear region in BMMs. Upon CSF-1 treatment, both BMM and RAW/LR5 cells massively reorganized their actin cytoskeleton resulting in the formation of F-actin-rich membrane protrusions. WAVE2 relocalized to this compartment as evidenced by the significant colocalization with F-actin (see lower panels in Fig. 2A,B). The accumulation of WAVE2 in CSF-1-elicited F-actin protrusions suggested a possible role for WAVE2 in the formation of these structures.

To investigate the possible involvement of WAVE2 in the formation of CSF-1 protrusions, WAVE2 activity or expression was inhibited in RAW/LR5 cells using different approaches, and the repercussion on the formation of F-actin rich membrane protrusions in response to CSF-1 was examined (Fig. 3). Initially, WAVE2 function was inhibited by expression of a FLAG-tagged WAVE2ΔV construct, in which the verprolin homology domain had been deleted, preventing the binding of G-actin and the subsequent activation of the Arp2/3 complex-mediated actin nucleation. This construct has been shown to act

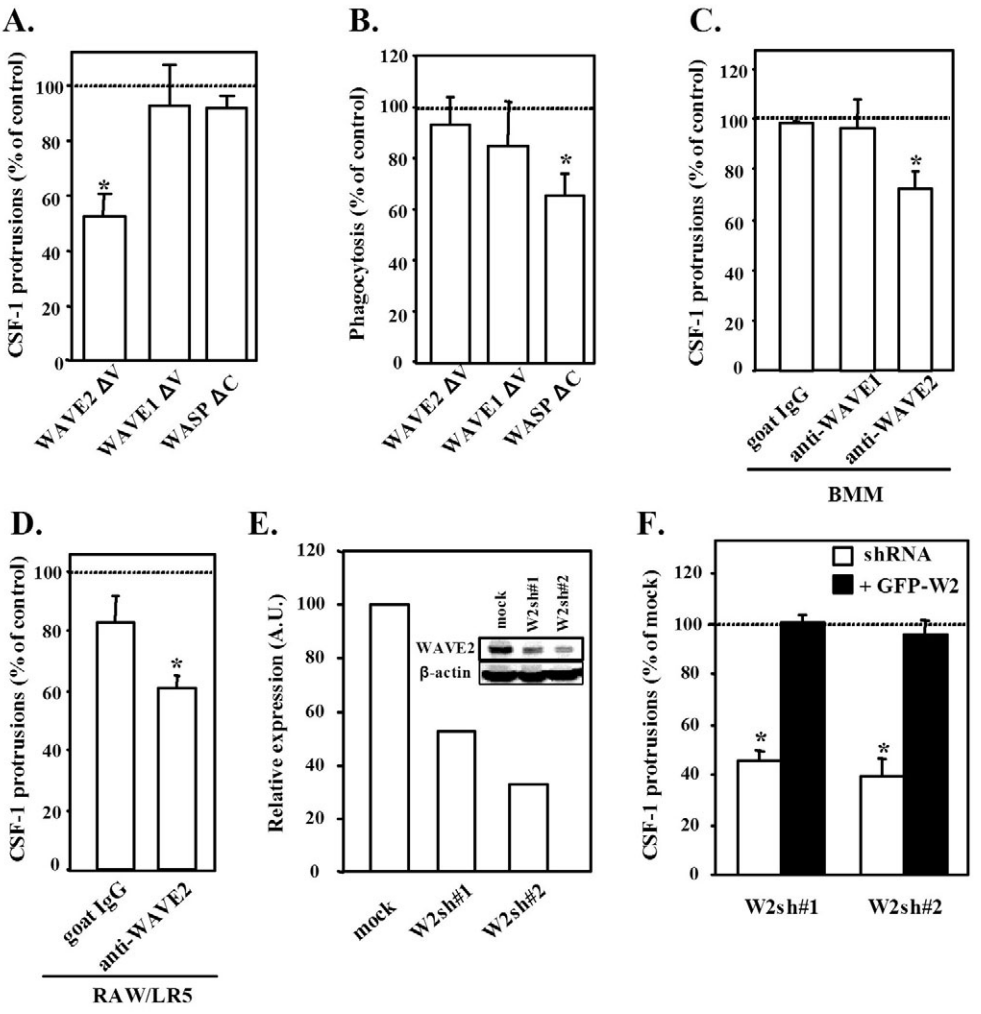

Fig. 3. Interfering with WAVE2 function inhibits CSF-1-induced F-actin rich membrane protrusions. (A) RAW/LR5 cells, transiently transfected with the indicated constructs, were treated with 20 ng/ml CSF-1 for 5 minutes and F-actin-rich protrusions were visualized by Alexa Fluor 568 phalloidin staining. Cells expressing the constructs were identified by epitope staining (FLAG for WAVE1/2 and Myc for WASP) and the number of CSF-1-elicited protrusions was quantified as described in Materials and Methods and expressed as a percentage of the CSF-1 stimulation observed in non transfected cells on the same coverslip; n=3, *P<0.05 compared to non transfected cells (represented by the dotted line in A-D). (B) RAW/LR5 cells were transiently transfected with the same constructs as in A, and their ability to undergo Fcγ-R-mediated phagocytosis was determined as described in Materials and Methods and expressed as a percentage of phagocytosis observed in non transfected cells on the same cover slip; n=3, *P<0.05 compared to non transfected cells. (C) WAVE1 or WAVE2 antibodies or control IgG were introduced into BMM cells by transient permeabilization as described in Materials and Methods. The ability of cells to form F-actin rich protrusions in response to CSF-1 was analyzed as in A; n=3, *P<0.05 compared to non permeabilized cells on the same coverslip. (D) WAVE2 antibodies or control IgG were introduced into RAW/LR5 cells by transient permeabilization, as in C, and CSF-1-induced protrusions were scored as in A; n=3, *P<0.05 compared to non permeabilized cells on the same cover slip. (E) WAVE2 and β-actin expression in WAVE2 shRNA-treated RAW/LR5 cells (heterogenous cell populations) was analyzed by western blotting with the appropriate antibodies and compared to mock shRNA treated cells (see inset) and WAVE2/β-actin signal intensity ratios of the indicated blot were quantified. (F) Mock, WAVE2 shRNA-treated cells (white bars) or WAVE2 shRNA-treated cells transiently transfected with a human GFP-WAVE2 construct (black bars) were stimulated with CSF-1 and their ability to form F-actin-rich protrusions in response to CSF-1 was analyzed as in A and expressed as a percentage of the CSF-1 stimulation observed in mock shRNA-treated cells; n=3, *P<0.05 compared to mock shRNA-treated cells.

as a dominant-negative mutant (Miki et al., 1998). Transfected cells were identified on the basis of their expression of the FLAG epitope and cells expressing FLAG-WAVE2ΔV showed reduced F-actin protrusions in response to CSF-1 compared to non transfected cells. In order to quantitatively evaluate this WAVE2ΔV effect, the average protrusion index of cells in response to CSF-1 was scored as described in Materials and Methods. In response to CSF-1, F-actin rich membrane protrusions were decreased by 50% in cells expressing the WAVE2ΔV mutant compared with non transfected cells (Fig. 3A). By contrast, expression of dominant-negative versions of WAVE1 (WAVE1ΔV, designed in a similar way to the WAVE2 mutant) and WASP (WASPΔC, in which the cofilin homology domain has been deleted) did not result in any significant reduction of CSF-1-induced F-actin-rich membrane protrusions. The effect of these three constructs was also evaluated with respect to another actin-dependent process in macrophages, namely Fcγ-R-mediated phagocytosis. The ability of cells expressing the different mutants to ingest IgG-opsonized erythrocytes was determined by comparison with non transfected cells. Expression of WAVE2ΔV and WAVE1ΔV mutants had no significant effect on the number of ingested particles per cell, whereas WASPΔC expression led to a 35% decrease in Fcγ-R-mediated phagocytosis. This result was in agreement with the reduced phagocytosis observed in WASP-deficient macrophages (Lorenzi et al., 2000). The expression level of WAVE1ΔV in individual RAW/LR5 cells was entirely comparable to the expression level of the WAVE2ΔV mutant, as determined by FLAG immunofluorescence measurement (data not shown). Since dominant-negative mutants may have non specific effects, WAVE2-specific antibodies were introduced into BMM or RAW/LR5 cells by transient permeabilization as described in Materials and Methods as an alternative way to block WAVE2 function. Consistent with the result obtained with the WAVE2ΔV construct, cells loaded with WAVE2-specific antibodies showed a significant decrease in the formation of F-actin-rich membrane protrusions in response to CSF-1 compared to non permeabilized cells, whereas cells loaded with control IgG (or WAVE1 antibodies in the case of BMM) were not significantly altered in their ability to form F-actin protrusions (Fig. 3C,D).

To confirm the results using dominant-negative mutants or blocking antibodies, endogenous WAVE2 expression was reduced using RNA-mediated interference (RNAi). Retroviral delivery of WAVE2-specific shRNA (W2sh#1 and W2sh#2, targeting two distinct regions of the WAVE2 transcript) in RAW/LR5 cells resulted in a 50-70% reduction of WAVE2 protein expression compared to mock shRNA-treated cells as determined by western blot analysis (Fig. 3D). No enhanced WAVE1 expression was detected in cells with reduced WAVE2 levels (data not shown). The ability of cells with reduced WAVE2 expression to exhibit F-actin rich membrane protrusions in response to CSF-1 was then evaluated and compared to mock shRNA-treated cells. Consistent with results shown in Fig. 3A,C, reduction of WAVE2 expression resulted in a significant inhibition of CSF-1-induced membrane protrusions (Fig. 3E). No difference was observed in the ability of mock shRNA-treated cells to extend protrusions in response to CSF-1 compared to non infected cells (not shown). Notably, re-expression of a human GFP-WAVE2 wild-type protein, which should not be affected by the mouse-specific shRNA, was able to fully rescue the ability of WAVE2 shRNA-treated cells to extend protrusions in response to CSF-1 (see black bars, Fig. 3E). Also, reduction of WAVE2 expression did not affect Fcγ-R-mediated phagocytosis, consistent with the lack of effect of the WAVE2ΔV construct shown previously (Fig. 3B; see supplementary material Fig. S1).

Therefore, using a variety of approaches, we demonstrated that the integrity of WAVE2 function and expression was necessary for the generation of F-actin-rich membrane protrusions in response to CSF-1 in RAW/LR5 cells.

Contribution of Abi1 to the regulation of WAVE2

Recently, several studies have shown that WAVE2 is present within the cells as part of a protein complex whose integrity

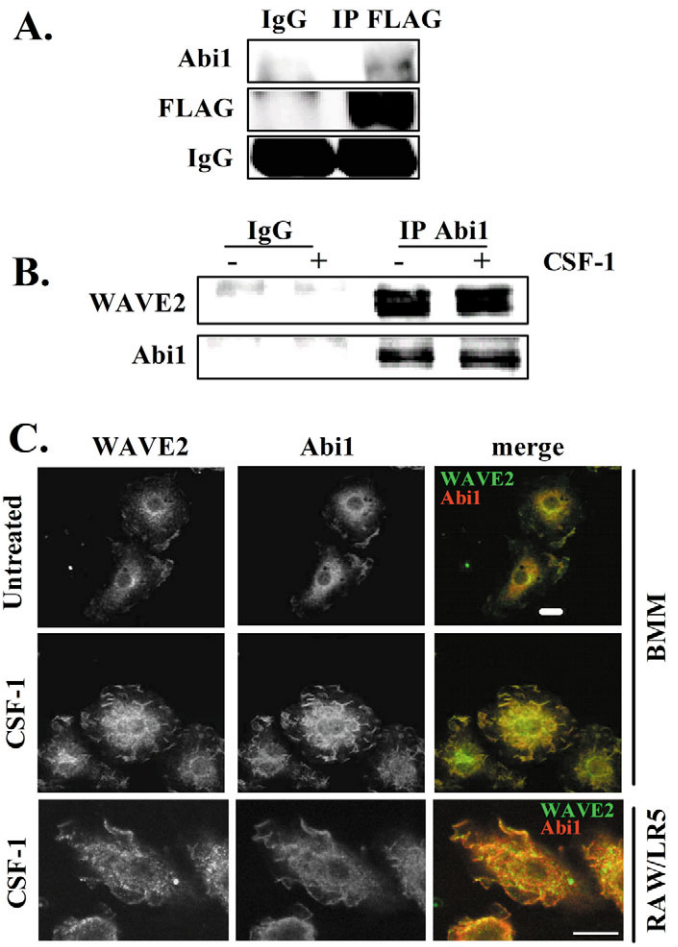

Fig. 4. WAVE2 and Abi1 are found in the same complex and are enriched at CSF-1-induced membrane protrusions. (A) FLAG-tagged WAVE2-expressing RAW/LR5 cells were lysed and lysates were sequentially incubated with control IgG and specific antibodies against FLAG for immunoprecipitation (IP). Immunoprecipitates were then subjected to western blotting using the indicated antibodies. Signals corresponding to IgG heavy chain are shown as a proof of equal loading. (B) RAW/LR5 cells were either left untreated (–) or were treated (+) with 20 ng/ml CSF-1 for 5 minutes prior to lysis and immunoprecipitation of Abi1 followed by WAVE2 and Abi1 western blotting. (C) BMM and RAW/LR5 cells were treated with CSF-1, then fixed and stained for WAVE2 and Abi1 as described in Materials in Methods. Bars, 10 μm.

was important for the activity and/or stability and/or localization of WAVE2. The exact components and function of this complex remain a matter of debate. Nevertheless, different reports have provided evidence for the existence of WAVE2 in a complex bound to the Abelson kinase interactor protein Abi1 in various cell types. To test whether a similar complex existed in macrophages, we performed reciprocal immunoprecipitations of WAVE2 and Abi1 from RAW/LR5 cell lysates. Since the commercially available WAVE2 antibodies did not efficiently immunoprecipitate WAVE2, RAW/LR5 cells were transiently transfected with a FLAG-tagged WAVE2 construct prior to lysis, followed by immunoprecipitation of WAVE2 using anti-FLAG antibody. Western blot analysis of FLAG immunoprecipitates using anti-Abi1 antibody indicated that Abi1 coimmunoprecipitated with FLAG-WAVE2 (Fig. 4A). No Abi1 signal was detected when control IgG was used for immunoprecipitation. In addition, immunoprecipitation of endogenous Abi1 from RAW/LR5 cell lysates also specifically immunoprecipitated endogenous WAVE2 (Fig. 4B). This indicated that WAVE2 and Abi1 were part of the same protein complex. Since different models have been proposed in which the WAVE-Abi complex is either dismantled (Eden et al., 2002) or remains constant (Innocenti et al., 2004) or is found in higher amounts (Leng et al., 2005) upon cell stimulation, we sought to evaluate the status of the WAVE2-Abi1 complex in macrophages in response to CSF-1. RAW/LR5 were left untreated or were treated with CSF-1 for 5 minutes prior to immunoprecipitation of endogenous Abi1. Similar amounts of WAVE2 were detected in Abi1 immunoprecipitates from both treated and non treated cells (Fig. 4B), suggesting that the WAVE2-Abi1 complex remains unchanged following CSF-1 treatment. To confirm this, WAVE2 and Abi1 subcellular localization was investigated by immunostaining of both proteins in BMM and RAW/LR5 cells. As shown in Fig. 4C, Abi1 mainly colocalized with WAVE2 in the perinuclear cytosol of resting BMM and, upon CSF-1 treatment, both Abi1 and WAVE2 were significantly enriched in membrane protrusions. A similar WAVE2-Abi1 colocalization was observed in RAW/LR5 cells (Fig. 4C, bottom panels). These observations are consistent with the existence of an active WAVE2-Abi1 complex, which does not dissociate and is recruited to specific sites upon CSF-1 stimulation.

In order to determine the functional importance of the integrity of the WAVE2-Abi1 complex in CSF-1-induced F-actin rich membrane protrusions, Abi1 expression was reduced using RNAi in RAW/LR5 cells. As previously performed with WAVE2, cells were retrovirally infected with two different shRNAs targeting two distinct regions of the Abi1 transcript (sh1 and sh2). Puromycin-resistant clonal populations were generated with varying levels of Abi1 expression compared to mock shRNA treated cells, as shown by western blot analysis (Fig. 5A). Notably, the reduction of Abi1 protein expression observed in different clones was systematically associated with a proportional reduction in the levels of WAVE2 (see

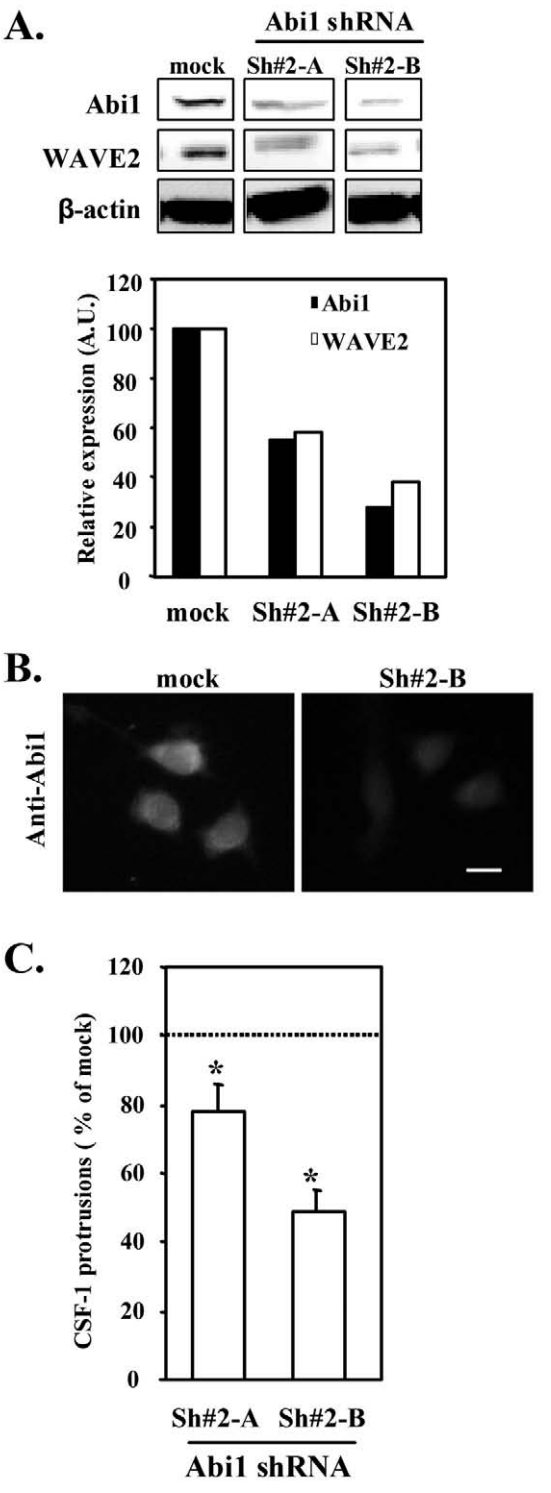

Fig. 5. Abi1 and WAVE2 are required for CSF-1-induced F-actin rich membrane protrusions in macrophages. (A) Abi1, WAVE2 and β-actin expression in Abi1 shRNA-treated cells (two different clonal populations shown) was analyzed by western blotting with the appropriate antibodies and compared to mock shRNA-treated cells. Quantification of Abi1/β-actin and WAVE2/β-actin signal intensity ratios is shown below. (B) Mock- and Abi1-shRNA-treated cells were fixed and stained for Abi1 as described in Materials and Methods. Bar, 10 μm. (C) Mock shRNA-treated cells and two independent clones with reduced Abi1/WAVE2 expression (Abi1 shRNA) were treated with 20 ng/ml CSF-1 for 5 minutes and the ability of cells to form F-actin-rich membrane protrusions in response to CSF-1 was scored as described in Fig. 3 and expressed as a percentage of the CSF-1 stimulation observed in mock shRNA-treated cells; n=3, *P<0.05 compared to mock shRNA-treated cells.

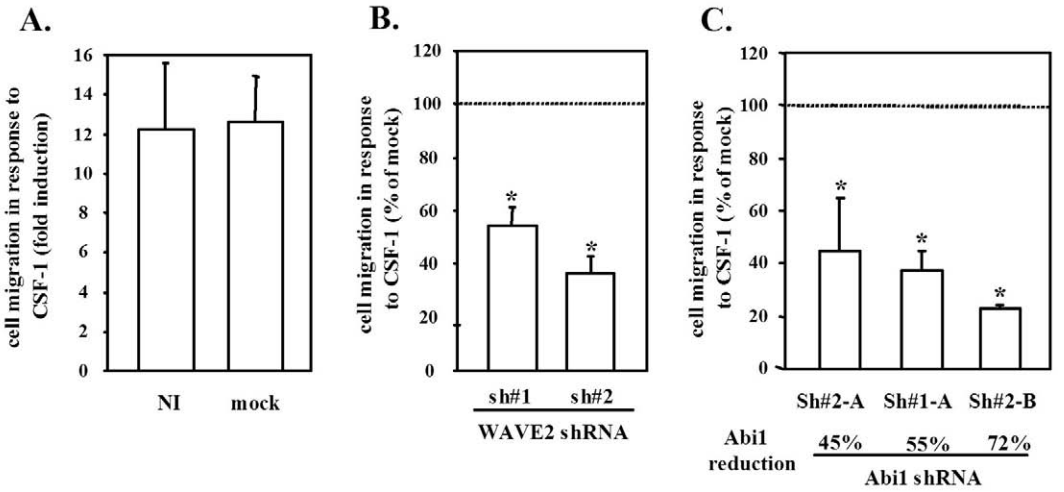

Fig. 6. WAVE2 and Abi1 are required for CSF-1-induced cell migration in macrophages. (A) Non infected (NI) and mock shRNA-treated cells showed no significant difference in cell migration in response to 20 ng/ml CSF-1 using a transmigration chamber assay as described in Materials and Methods, n=4. (B,C) Using this assay, cell migration in response to CSF-1 was determined in WAVE2 shRNA-treated cells (B) or Abi1 shRNA-treated cells (C) and compared with that of mock shRNA-treated cells. CSF-1-stimulated migration of each cell population was compared with the corresponding unstimulated condition and expressed as a percentage of the mock shRNA-treated cells; n=3 for B and C, *P<0.05 compared to mock shRNA-treated cells.

quantification, Fig. 5A). Reduction of Abi1 expression was also analyzed by immunofluorescence microscopy with a degree of extinction comparable to that determined using western blot (Fig. 5B). The ability of cells with reduced Abi1, and therefore WAVE2, expression to exhibit F-actin-rich membrane protrusions in response to CSF-1 was then evaluated and compared to mock shRNA-treated cells. Consistent with our previous results obtained following WAVE2 reduction (see Fig. 3E), Abi1 shRNA-treated cells showed a significant inhibition of CSF-1-induced membrane protrusions compared to mock shRNA-treated cells (Fig. 5C). Importantly, cells with the greatest reduction of Abi1/WAVE2 expression showed the greatest inhibition of CSF-1-induced protrusions, suggesting a direct correlation between the level of reduction of Abi1/WAVE2 and the ability of cells to respond to CSF-1.

Finally, we sought to test whether the defect in extending protrusions in response to CSF-1 observed in cells treated with either WAVE2 shRNA or Abi1 shRNA was associated with an impaired ability of cells to migrate towards CSF-1. Cells were subjected to a transmigration chamber assay with 20 ng/ml CSF-1 being added in the lower chamber. A ~12-fold increase in cell migration in response to CSF-1 was observed in both non infected (NI) and mock shRNA-treated cells (Fig. 6A). However, WAVE2 shRNA-treated cells were significantly impaired in their ability to migrate in response to CSF-1 compared to mock shRNA-treated cells, with the greatest reduction of cell migration observed in the cells having the lowest expression of WAVE2 (Fig. 6B). Abi1 shRNA-treated cells also showed a marked reduction in migration in response to CSF-1, with, again, the greatest inhibition obtained in the cells showing the lowest levels of Abi1 and WAVE2 proteins (Fig. 6C). This effect on cell migration closely paralleled the apparent dose-dependent inhibition of CSF-1-induced protrusions observed in the different clones with variable levels of Abi1 and WAVE2 proteins. Therefore, WAVE2, in a WAVE2-Abi1 complex, is required for macrophage migration towards CSF-1.

Taken together, our data identify a functional role for the WASP family member WAVE2, through the WAVE2-Abi1 complex, in mediating the formation of F-actin-rich membrane protrusions and cell migration in response to CSF-1 in macrophages.

Discussion

In different cell types, WAVE proteins have been shown to play a significant role in the remodeling of the actin cytoskeleton through their ability to stimulate Arp2/3 complex-dependent actin assembly. However, no information was available with respect to the function of WAVEs in macrophages and our study is the first to document this issue. Analysis of the expression profile of WAVE mRNAs in human and mouse revealed that WAVE1 was expressed mostly in the brain but could also be found in other tissues including lung, liver, kidney and heart, WAVE2 was almost ubiquitously distributed with a strong expression in peripheral leukocytes and WAVE3 was mostly restricted to the brain (Sossey-Alaoui et al., 2003; Suetsugu et al., 1999). Here, we found that WAVE2 was significantly expressed at the protein level in macrophages of human and mouse origin, whereas WAVE1 was detected at extremely low levels and only in the RAW/LR5 cell line. This observation is in agreement with the finding that WAVE1 protein expression was decreased upon differentiation of promyeloid cells into monocyte/macrophage-like cells (Launay et al., 2003).

The differential expression of WAVEs in cells and tissues raises the question of whether the three isoforms have distinct functions or are partially redundant. Our results show that inhibition of WAVE2 function through a variety of approaches impairs the ability of macrophages to generate F-actin-rich membrane protrusions in response to CSF-1. Interestingly, a dominant-negative mutant of WAVE1 – similar to the specific inhibitory construct used for WAVE2 – had no effect on this

process, supporting the idea of a specific involvement of WAVE2 in CSF-1-induced protrusion formation and arguing against the possibility of a functional role of a very small amount of WAVE1 that could be functionally relevant in macrophages. Several lines of evidence suggest that WAVEs might have different functions. First, the very different phenotypes resulting from WAVE1 or WAVE2 gene invalidation in mice clearly indicate differences in the functional importance of these two isoforms and reflect their partially distinct expression in tissues. Indeed, WAVE2 deficiency results in embryonic lethality during mid-gestation, probably due to defects in various aspects of cell migration required for proper embryogenesis (Yamazaki et al., 2003; Yan et al., 2003), whereas WAVE1 disruption has a less severe effect, resulting primarily in central nervous system dysfunction (Dahl et al., 2003; Soderling et al., 2003). Second, studies directly comparing WAVE1 and WAVE2 function in the same cells also indicate differences in their subcellular localization (Nozumi et al., 2003), their relative affinities for specific proteins important for their regulation (Oda et al., 2004) and, most notably, Suetsugu and colleagues have shown that WAVE1 and WAVE2 are each required for the formation of discrete F-actin-rich structures that are induced in fibroblasts upon platelet-derived growth factor (PDGF) treatment (Suetsugu et al., 2003).

In macrophages, CSF-1 treatment induces the formation of dorsolateral F-actin-containing membrane projections (also called ruffles). We showed here that WAVE2 was relocalized to this compartment upon cell stimulation and, using three different approaches, we demonstrated that WAVE2 function was required for the full macrophage ruffling response to CSF-1. Importantly, inhibition of the CSF-1-induced WAVE2-dependent cytoskeletal rearrangement also resulted in a reduced ability of cells to migrate toward CSF-1. Altogether these data identify WAVE2 as a major mediator of macrophage motility in response to CSF-1. The cell signaling from the activated CSF-1 receptor to the WASP/WAVE-mediated actin remodeling has not been entirely characterized, although independent studies have provided substantial evidence to describe parts of this pathway. For example, it has been shown that WAVE proteins can be found in a complex with and act downstream of the Rho GTPase Rac (Miki et al., 1998). Expression of a constitutively active form of Rac in cells results in WAVE translocation from cytosol to Rac-induced membrane protrusions, and disruption of WAVEs, using dominant-negative mutants or RNAi, inhibits the ability of Rac to induce such structures (Kurisu et al., 2005; Miki et al., 1998). Interestingly, Rac has been shown to be rapidly activated upon CSF1 treatment in macrophages with kinetics consistent with a concomitant or subsequent recruitment of WAVE2 into CSF-1-elicited membrane protrusions (Abell et al., 2004) (D.C., unpublished data). Moreover, CSF-1-induced actin remodeling is Rac-dependent in macrophages (Allen et al., 1997; Cox et al., 1997) and RacQ61L-induced membrane protrusions in RAW/LR5 cells are inhibited by expression of a WAVE2ΔV mutant (data not shown). A candidate of choice for being an upstream regulator of this putative Rac-WAVE2 interaction is PI 3-kinase, which is a known CSF-1R effector required for cell migration (Vanhaesebroeck et al., 1999) and which can activate different Rho GTPases including Rac (Ridley, 2001). A PI 3-kinase-WAVE2 link is further supported by the fact that the WAVE2-dependent actin remodeling in hepatocyte growth factor (HGF)-treated C2C12 myoblasts has also been shown to be PI 3-kinase-dependent (Kawamura et al., 2004). In total, these studies support the existence of a CSF-1R→PI3-kinase→Rac→WAVE2→actin pathway critical for macrophage migration. Obviously, further studies are required to unambiguously verify and delineate the complete CSF-1R to WAVE2 signaling cascade.

Unlike WASP and N-WASP, WAVE proteins are devoid of a canonical GTPase-binding domain and accordingly have never been reported to bind directly to Rac. For this reason, understanding how WAVEs can relay signals from activated Rac to the actin cytoskeleton represents a current area of active investigation and debate. The first protein described as a potential link between GTP-bound Rac and WAVE2 was the insulin receptor substrate IRSp53, which was found to stimulate WAVE2-dependent activation of Arp2/3 in vitro (Miki et al., 2000). Another study reported the binding of IRSp53 to Cdc42, rather than Rac, in a complex with the Ena/VASP family protein Mena (Krugmann et al., 2001), whereas work from a third group showed a subcellular colocalization of WAVE2 and IRSp53 independent of Mena (Nakagawa et al., 2003). The relevance of the IRSp53/WAVE2 interaction therefore remains unclear. More recently, a number of studies provided evidence for the existence of WAVE1/2 in a different multi-protein complex including Abi together with Nap1 and PIR121/Sra1 in mammalian cells (Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Steffen et al., 2004). Similar complexes were also identified in *Dictyostelium discoideum* and *Drosophila melanogaster* suggesting an evolutionarily conserved molecular organization (Blagg et al., 2003; Kunda et al., 2003; Rogers et al., 2003). Despite this apparent consensus, contrasting models have been proposed to explain how WAVE activity could be regulated in this context. The initial proposal was that WAVE1 would be kept inactive in this multi-protein complex and, following cell stimulation and binding of activated Rac to PIR121, the complex would dissociate, resulting in the release of active WAVE1 (Eden et al., 2002). Subsequently, another report suggested that the WAVE2-containing complex remained intact when bound to Rac and was relocalized at active sites of actin assembly within the cell (Innocenti et al., 2004). The details of the regulation of WAVE function have not been fully delineated and this issue awaits further investigations. In macrophages, we clearly demonstrated that WAVE2 and Abi1 were found in the same molecular complex and that both proteins were recruited to and necessary for CSF-1-induced F-actin-rich protrusions. This, therefore, expands the list of cell types where a WAVE-Abi complex is functionally relevant to couple extracellular stimuli to actin rearrangement. Interestingly, disruption of the WAVE2-Abi1 complex by reducing the expression of Abi1 in macrophages was associated with a proportional reduction in WAVE2 protein levels, consistent with a previous report indicating that, when released from its stabilizing complex, WAVE/Scar is targeted to proteolytic degradation (Kunda et al., 2003). Furthermore, cells with reduced amounts of WAVE2 alone (generated using WAVE2-specific shRNAs) were virtually indistinguishable from cells with reduction of both WAVE2 and Abi1 (obtained by targeting Abi1 exclusively), in terms of residual WAVE2 levels, number of membrane protrusions and cell migration in response to CSF-1. This

observation suggests that the WAVE2-Abi1 complex represents a major functional pool of WAVE2 in macrophages. Taken together, these data are in favor of a CSF-1-induced relocalization of the entire preformed WAVE2-Abi1-containing complex to specific subcellular sites where it can stimulate actin assembly. Our results, however, do not formally rule out the possibility of additional levels of WAVE2 regulation, including potential interactions with IRSp53, phosphatidylinositol (3,4,5)-trisphosphate and/or phosphorylation of residues, all possibly important for its activity and localization, as previously described (Leng et al., 2005; Miki et al., 1999; Miki et al., 2000; Oikawa et al., 2004).

In conclusion, our study identifies, for the first time, WAVE2 as a critical component of CSF-1-induced macrophage migration, presumably through its ability to polymerize actin and to contribute to the production of membrane protrusions in response to the chemoattractant. We also provide evidence that WAVE2 functions within a complex with Abi1 and that the integrity of this molecular association is required for an optimal migratory response of macrophages.

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