Evidence for a molecular complex consisting of Fyb/SLAP, SLP-76, Nck, VASP and WASP that links the actin cytoskeleton to Fcγ receptor signalling during phagocytosis

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SUMMARY

Phagocytosis by macrophages and neutrophils involves the spatial and temporal reorganisation of the actin-based cytoskeleton at sites of particle ingestion. Local polymerisation of actin filaments supports the protrusion of pseudopodia that eventually engulf the particle. Here we have investigated in detail the cytoskeletal events initiated upon engagement of Fc receptors in macrophages. Ena/vasodilator-stimulated phosphoprotein (VASP) proteins were recruited to phagosomes forming around opsonised particles in both primary and immortalised macrophages. Not only did the localisation of Ena/VASP proteins coincide, spatially and temporally, with the phagocytosis-induced reorganisation of actin filaments, but their recruitment to the phagocytic cup was required for the remodelling of the actin cytoskeleton, extension of pseudopodia and efficient particle internalisation. We also report that SLP-76, Vav and profilin were recruited to forming phagosomes. Upon induction of phagocytosis, a large molecular complex, consisting in part of Ena/VASP proteins, the Fyn-binding/SLP-76-associated protein (Fyb/SLAP), Src-homology-2 (SH2)-domain-containing leukocyte protein of 76 kDa (SLP-76), Nck, and the Wiskott-Aldrich syndrome protein (WASP), was formed. Our findings suggest that activation of Fcγ receptors triggers two signalling events during phagocytosis: one through Fyb/SLAP that leads to recruitment of VASP and profilin; and another through Nck that promotes the recruitment of WASP. These converge to regulate actin polymerisation, controlling the assembly of actin structures that are essential for the process of phagocytosis.

Key words: Actin cytoskeleton, Ena/VASP proteins, Profilin, Phagocytosis, Arp2/3 complex

INTRODUCTION

Phagocytosis, a central component of the innate immune response, is the process whereby specialised cell types recognise and engulf foreign extracellular material. Recognition and binding of foreign material by neutrophils and macrophages is assisted by coating of particles with opsonins such as complement and immunoglobulins (Ig) (Brown, 1995; Allen and Aderem, 1996b). Ig-coated particles are recognised by the Fc family of cell surface receptors (FcR), and binding to these receptors induces a cascade of intracellular signalling events that triggers the ingestion of particles (Indik et al., 1995; Allen and Aderem, 1996b; Greenberg, 1999). The phagosome then undergoes a maturation process that involves interactions with other intracellular compartments and culminates in the formation of a phagolysosome where the ingested particle is destroyed (Desjardins et al., 1994; Beron et al., 1995; Allen and Aderem, 1996b).

As an integral part of FcR-mediated phagocytosis, the remodelling of the actin cytoskeleton supports the extension of pseudopodia at sites of particle engulfment (Greenberg et al., 1990; Maniak et al., 1995; Allen and Aderem, 1996a). Currently, much research is focused on describing the signalling pathways triggered by FcR ligation and that initiate actin cytoskeleton reorganisation. Ligation and clustering of the Fcγ receptor by IgG stimulates Src-family kinases to phosphorylate the immunoreceptor tyrosine-based activation motif (ITAM) of the FcγR (Greenberg et al., 1993; Greenberg et al., 1994). ITAM phosphorylation induces recruitment of several cytoplasmic enzymes, including the tyrosine kinase Syk (Greenberg et al., 1994), phosphatidyl inositol 3-kinase (PI 3-K) (Araki et al., 1996; Cox et al., 1999), phospholipase C (PLC) (Seastone et al., 1999), the Src homology 2 (SH2)-containing 5′ inositol phosphatase (SHIP) (Maresco et al., 1999), the adapter proteins SH2-domain-containing leukocyte protein of 76 kDa (SLP-76) (Bonilla et al., 2000), B cell linker protein (BLNK) (Bonilla et al., 2000) and linker of activation of T cell (LAT) (Tridandapani et al., 2000). However, the chain of molecular events that specifically links Fc receptor ligation to actin reorganisation has not been elucidated.

Much has been learned about the remodelling of the actin cytoskeleton in mammalian systems through study of the well characterised mechanism of actin-based motility in *Listeria monocytogenes*. This bacterium invades mammalian cells, entering their cytosol, where it propels itself by developing a comet-like, actin-rich tail (Tilney and Portnoy, 1989; Sechi et al., 1997). Generation of the actin tail by *Listeria* is due exclusively to the expression of ActA on their surface (Domann et al., 1992; Kocks et al., 1992). Two major domains of ActA are essential for its function: a four-fold E/DFPPPPXDEE motif (ActA repeats) and an amino-terminal stretch of positively charged amino acids. The ActA repeats bind with high affinity to proteins of the Ena/VASP family (Chakraborty et al., 1995; Niebuhr et al., 1997), which are necessary for efficient *Listeria* motility both in cells and in cell-free extracts (Smith et al., 1996; Laurent et al., 1999; Loisel et al., 1999), suggesting that they function as regulators of the actin cytoskeleton. VASP also localises at the front of spreading lamellipodia, where its levels correlate with the speed of lamellipodia protrusion (Rottner et al., 1999). In addition, Mena, another member of this family, is highly concentrated at the distal tips of growth cone filopodia, and a neuronal-specific Mena isoform induces the formation of actin-rich cell surface projections in fibroblasts (Gertler et al., 1996; Lanier et al., 1999). Moreover, VASP and Mena are ligands for profilin (Reinhard et al., 1995; Gertler et al., 1996; Kang et al., 1997), an actin-monomer-binding protein that, under favourable conditions, stimulates the polymerisation of actin (Pantaloni and Carlier, 1993) and whose recruitment to the *Listeria* surface directly correlates with the motility state of this bacterium (Geese et al., 2000).

Another cellular ligand of ActA that has recently attracted much attention is the Arp2/3 complex. This seven-protein complex localises to the lamellipodia of cultured cells and to the actin comet tails of *Listeria monocytogenes* and is essential for the actin-based motility of this bacterium (Kelleher et al., 1995; Machesky et al., 1997; Welch et al., 1997a; Welch et al., 1997b). The Arp2/3 complex interacts with the N-terminus of ActA and promotes the nucleation of actin filaments that is enhanced upon interaction with ActA (Welch et al., 1998; May et al., 1999; Pistor et al., 2000; Skoble et al., 2000; Zalevsky et al., 2000). These findings clearly indicate that Ena/VASP proteins and the Arp2/3 complex are key players in the actin-based processes.

The remodelling of the actin cytoskeleton is also essential for T-cell activation (Penninger and Crabtree, 1999). We have recently demonstrated that Fyb/SLAP is a new ligand for Ena/VASP proteins and that it localises to the sites of T cell receptor (TCR) clustering when T cells interact with anti-CD3-coated beads, a process that mimics the interaction with antigen-presenting cells (Krause et al., 2000). We also provided evidence for a model in which Ena/VASP proteins and the Arp2/3 complex are linked to the T-cell-bead interface via a molecular complex formed by SLP-76, Nck and WASP (Krause et al., 2000). In a physiological context, the formation of this molecular complex was proposed to be essential for the targeting of Ena/VASP proteins and the Arp2/3 complex and thus the initiation of early events that are essential for the remodelling of the actin cytoskeleton in T cell activation.

On the basis of these findings, it is conceivable that Fyb/SLAP and Ena/VASP proteins participate in the actin assembly process triggered by FcγR crosslinking during phagocytosis in macrophages. We present evidence that both proteins localise to nascent phagosomal cups and that a molecular complex formed by SLP-76, Nck and WASP is necessary for actin reorganisation during particle engulfment.

MATERIALS AND METHODS

Reagents and antibodies

Cell culture media and fetal bovine serum were purchased from Wisent Inc. HEPES-buffered RPMI and human IgG were obtained from Sigma. Sheep red blood cells (RBC) and rabbit anti-sheep RBC were purchased form ICN-Cappel. Latex beads were purchased from Bangs Laboratories. The following primary antibodies were used: Evl [monoclonal 84H1 (Lanier et al., 1999)], Fyb/SLAP (Krause et al., 2000), Nck (monoclonal #N15920; Transduction Laboratories), SLP-76 (monoclonal #S60720; Transduction Laboratories), zyxin [monoclonal 164D4 (Krause et al., 2000)], VASP (Krause et al., 2000), Vav [monoclonal Vav-30 (Sattler et al., 1995)], WASP [polyclonal Fus3 (Symons et al., 1996)] and WASP [monoclonal 67B4 (Krause et al., 2000)]. Alexa 488- and Alexa 594-conjugated secondary antibodies and rhodamine-phalloidin were purchased from Molecular Probes. CY2-conjugated goat anti-mouse IgG was purchased from Dianova.

GFP-tagged proteins and antibody production

Fyb/SLAP 1 was cloned into the pEGFP-N1 vector (Clontech) using *Sal*I and *Bam*HI. The green fluorescent protein (GFP)-VASP construct, the GFP-tagged ActA repeats (wild-type and mutated variant) and the GFP-tagged profilin II have already been described (Carl et al., 1999; Geese et al., 2000; Krause et al., 2000). The polyclonal antibody #80 was generated against a fragment encoding the C-terminus of murine Fyb/SLAP2 [clone 5/7 (Krause et al., 2000)]. This fragment was cloned into the pGEX-2TK (Pharmacia) using *Nco* and *Eco*RI. The GST-tagged purified protein was then used to immunise rabbits. The antiserum #80 was affinity purified using the same fragment cloned into the pGEX-6P1 vector, digested with Prescission™ protease (Pharmacia) to remove the GST moiety and immobilised on EAH-sepharose (Pharmacia).

Cell culture and transfection

Human macrophages were derived in vitro from peripheral blood monocytes. Briefly, 60 ml of fresh human blood was obtained by venipuncture and mixed with heparin. This was mixed with 9 ml of a 4.5% dextran/0.9% saline solution (final dextran concentration, 0.7%) and allowed to stand at room temperature for 45 minutes. The supernatant was removed, layered onto Ficoll-Hypaque solution, and the samples were centrifuged at 3000 rpm for 20 minutes. The mononuclear cell layer was removed and the cells were washed three times with sterile, cold medium RPMI 1640 and counted. 2.5×10^5 cells were plated onto 25 mm glass coverslips and incubated in RPMI 1640 supplemented with 10% FBS at 37°C under 5% CO_2. After three or four days, the non-adherent lymphocytes were removed and the cells were cultured for an additional five to 14 days at which point differentiated macrophages had adhered to and spread on the coverslips.

Raw264.7 mouse macrophages were grown in alpha-MEM supplemented with 10% FCS at 37°C and 7% CO_2. Raw264.7 cells (2.25×10^6 cells re-suspended in serum-free alpha-MEM supplemented with 20 mM HEPES) were transfected with 40 μg of DNA by electroporation using a Gene Pulser II (Bio-Rad) with the following settings: voltage, 0.17 kV; capacitance, 1000 μF.

Phagocytosis assays

Sheep RBC were opsonised with rabbit anti-sheep RBC antibody

(1:50). 3 μm latex beads were opsonised with 1 mg/ml human IgG. Opsonisation was performed at 37°C, for one hour followed by three washes with PBS. For samples that were to be analysed by confocal microscopy, cells were incubated with opsonised sheep RBC and then washed with PBS to remove non-bound sheep RBC. The samples were fixed and processed for immunocytochemistry as described below. For time-lapse imaging of live macrophages, cells growing on coverslips were placed in Leiden microperfusion chambers and bathed in HEPES-buffered RPMI medium. The samples were placed in a heated stage mount, warmed to 37°C and overlaid with opsonised sheep RBC (~50/macrophage). DIC and GFP fluorescence images were monitored simultaneously using a Leica inverted microscope (model DMIRB) with a 100× oil-immersion objective and appropriate filter sets. Images were captured using a cooled CCD camera (Princeton Instruments Inc.) driven by Metamorph software (Universal Imaging).

Immunofluorescence microscopy and image analysis

Cells were fixed and immunolabelled according to the methods described in (Geese et al., 2000). Specimens were observed using an Axiovert 135 TV microscope (Zeiss) equipped with a Plan-Apochromat 100×/1.40 NA oil immersion objective in combination with 1.6× or 2.5× optovar optics. Images were recorded with a cooled, back-illuminated CCD camera (TE/CCD-1000 TKB, Princeton Instruments Inc.) driven by IPLab Spectrum software (Scanalytics Inc.). For confocal microscopy, samples were analysed with a Zeiss LSM 510 laser scanning confocal microscope under a 100× oil immersion. Digital handling of the images was done using IPLab Spectrum, MicroTome (Vaytek, Inc.), Adobe Photoshop 5.0 (Adobe Systems, Inc.) and CorelDraw 8.0 (Corel Corp.).

Scanning electron microscopy

For scanning electron microscopy (SEM), Raw264.7 cells were transiently transfected with wild-type or mutated GFP-ActA repeats and sorted using a FACS sorter (FACS Vantage, Becton Dickinson). This approach was necessary to obtain a cell population expressing equivalent levels of GFP-tagged proteins and facilitated the analysis of the cells by SEM. After the phagocytosis assay, Raw264.7 cells were fixed with 4% PFA in cytoskeleton buffer (10 mM PIPES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM $MgCl_2$, pH 7.0) for 30 minutes at room temperature, then washed with the CB buffer. Cells were post-fixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate, 0.09 M sucrose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, pH 7.2) for two hours at room temperature. After washing with cacodylate buffer, the cells were dehydrated through a graded series of ethanol, processed by critical point drying and gold coated. Samples were examined with a digital scanning electron microscope (DSM 982 Gemini, Zeiss) using a working distance of 2-4 mm and acceleration voltage of 5 Kv. Electron microscopy images were processed using Photoshop 5.0 (Adobe Systems, Inc.).

Stimulation of Raw264.7 cells and immunoprecipitation

To characterise the multimolecular complex involved in phagocytosis, Raw264.7 cells (5×10^7 cells/ml) were incubated with purified rat anti-mouse CD16/CD32 (clone 2.4G2 at final concentration of 5 μg/ml; PharMingen International) on ice for 30 minutes. Cells were then washed and incubated with $F(ab')_2$ goat anti-rat IgG (at final concentration of 20 μg/ml; Jackson ImmunoResearch) for three minutes at 37°C. Cell lysates were prepared in ice-cold NP-40 buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40), supplemented with proteases inhibitors (60 μg/ml chymostatin, 10 μg/ml pepstatin, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM Pefabloc), 1 mM Na_3VO_4 and 10 mM NaF. Untreated Raw264.7 cells were used as a negative control.

Immunoprecipitations were performed using immobilised antibodies, which were prepared by covalent binding of the WASP monoclonal antibody 67B4 to CNBr-sepharose (Pharmacia). As a negative control for immunoprecipitations, unspecific IgG purified from mouse ascites (#50334; ICN Biomedicals) were coupled to CNBr-sepharose. Immunoprecipitates were resolved by SDS-PAGE. As a positive control, Raw264.7 cell lysates were used. Western blots were processed using the ECL+ enhanced chemiluminescence detection kit (Amersham).

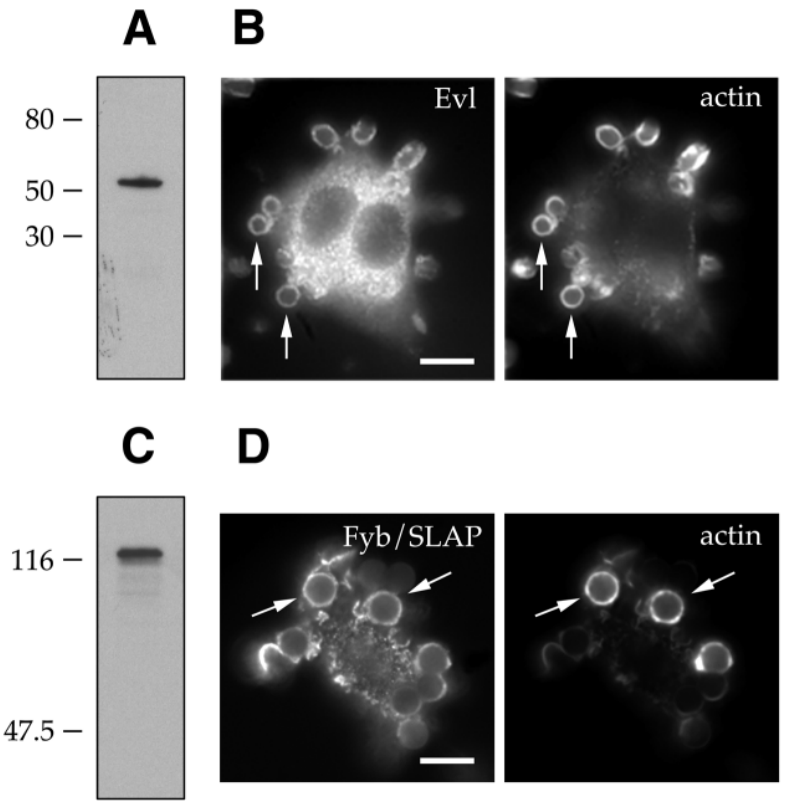

Fig. 1. Ena/VASP proteins and Fyb/SLAP accumulate at sites of phagocytosis in Raw264.7 macrophages. (A,C) Analysis by western blotting of protein extracts of Raw264.7 macrophages using an anti-Evl monoclonal antibody (A) and an anti-Fyb/SLAP affinity-purified polyclonal antibody (C). (B,D) Localisation of Evl and Fyb/SLAP in Raw264.7 macrophages during phagocytosis. Raw264.7 cells were incubated with IgG-opsonised sheep RBC (for Evl labelling) or IgG-coated beads (for Fyb/SLAP labelling) for approximately five minutes, fixed and stained with the above mentioned antibodies. Both Evl (B, left pa nel) and Fyb/SLAP (D, left panel) localise to phagocytic cups where they colocalise with actin (B,D; right panels). Arrows point to examples of phagocytic cups surrounding sheep RBC (B) or beads (D). Scale bar: 10 μm.

RESULTS

Ena/VASP proteins and Fyb/SLAP accumulate at sites of F-actin assembly around nascent phagosomes

To test the hypothesis that Ena/VASP proteins play a role in the actin-based processes associated with phagocytosis, we first examined their intracellular distribution during

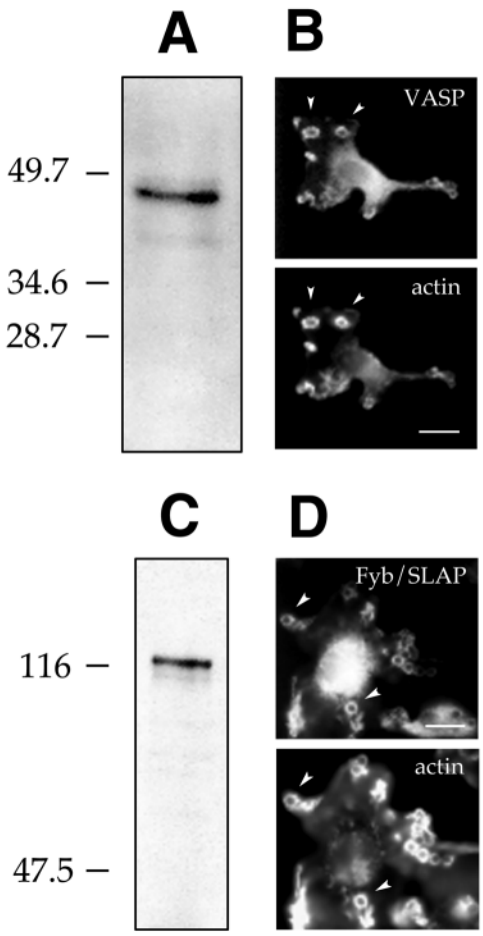

Fig. 2. Ena/VASP proteins and Fyb/SLAP accumulate at sites of phagocytosis in primary macrophages. (A,C) Western blot analysis of protein extracts of monocyte-derived macrophages using an anti-VASP monoclonal antibody (A) and anti-Fyb/SLAP (C) affinity-purified polyclonal antibody. (B,D) Analysis of VASP and Fyb/SLAP intracellular distribution in monocyte-derived macrophages during phagocytosis. Cells undergoing phagocytosis were incubated with IgG-opsonised sheep RBC (for VASP labelling) or beads (for Fyb/SLAP labelling) for approximately five minutes, fixed and stained with the antibodies described above. In macrophages engaged in phagocytosis, both VASP (B) and Fyb/SLAP (D) localise to forming phagosomes. Again, both proteins colocalise with actin at these sites (B,D). Arrows point to examples of phagocytic cups surrounding sheep RBC (B) or beads (D). Scale bar: 10 μm.

internalisation of opsonised sheep RBCs. For this purpose, we used a monoclonal antibody against Evl, a member of the Ena/VASP family that is highly enriched in heamatopoietic cells (Lanier et al., 1999). When tested with extracts from Raw264.7, this antibody reacted against a protein of the expected molecular weight (Fig. 1A), indicating that it is a reliable tool for analysing the distribution of Ena/VASP proteins in these cells.

We next examined the distribution of Evl in Raw264.7 cells undergoing phagocytosis of opsonised sheep RBCs. Within five minutes of adding sheep RBC to macrophages, we observed the formation of actin-rich cups around forming phagosomes, the hallmark of the phagocytic process (Fig. 1B, left panel). Within the same time, Evl localised at the phagocytic cups (Fig. 1B, left panel), where it overlapped with actin. These findings suggest that Ena/VASP proteins are involved in the Fcγ-receptor-mediated reorganisation of the actin cytoskeleton.

On the basis of the observations that Ena/VASP proteins localise to the actin-rich cups during phagocytosis, we sought to determine the molecular mechanism by which these proteins are targeted to the membrane of forming phagosomes. We have recently shown that Fyb/SLAP, a protein expressed exclusively in haematopoietic cells, co-localises with F-actin at sites of interaction between T cells and anti-CD3-coated beads (Krause et al., 2000). This process mimics the interaction of T-cells with antigen-presenting cells and is accompanied by the formation of lamellipodia-like cellular extensions in much the same way that they occur during phagocytosis. Because Fyb/SLAP binds to the EVH1 domain of Ena/VASP proteins (Krause et al., 2000), it could therefore play a role in actin assembly at the phagosome.

To assess the expression of Fyb/SLAP in Raw264.7 macrophages, protein extracts of these cells were tested by immunoblotting with an anti-Fyb/SLAP polyclonal antibody. This affinity-purified polyclonal antibody detected a single prominent band, which corresponds to the predicted molecular size of Fyb/SLAP (Fig. 1C), indicating that Raw264.7 cells indeed express this protein. We then analysed the intracellular distribution of Fyb/SLAP during phagocytosis in Raw264.7 cells. Detection of Fyb/SLAP with this affinity-purified polyclonal antibody precluded the use of sheep RBC opsonised with a rabbit antiserum in these experiments. Instead, phagocytosis was studied with human IgG-opsonised beads. In agreement with our findings above, Fyb/SLAP, like Ena/VASP proteins, localised at forming phagosomes within five minutes of the addition of the beads (Fig. 1D, left panel) and its distribution coincided with that of actin at the same sites (Fig. 1D, right panel). These observations indicate that Fyb/SLAP is involved in the actin-based process during phagocytosis and suggest that it might be responsible for recruitment of Ena/VASP proteins to phagocytic cups.

Ena/VASP proteins and Fyb/SLAP are expressed in monocyte-derived macrophages and accumulate at sites of F-actin assembly around nascent phagosomes

To corroborate the above findings, we analysed VASP and Fyb/SLAP expression in *bona fide* professional phagocytes derived from cultures of peripheral blood monocytes from human donors. Adherent monocyte-derived macrophages were harvested and 10 μg of total cellular protein was analysed by SDS-PAGE and immunoblotted with an anti-VASP monoclonal antibody or with an anti-Fyb/SLAP polyclonal antibody. The antibodies detected bands corresponding to the molecular weights of VASP and Fyb/SLAP (Fig. 2A,C), thus indicating that these proteins are expressed at detectable levels in professional macrophages.

We next examined the intracellular distribution of VASP in monocyte-derived macrophages ingesting IgG-opsonised sheep RBC. Again, within five minutes of addition of sheep RBC to macrophages, VASP accumulated at, and co-localised with, actin at forming phagosomes (Fig. 2B). The distribution of Fyb/SLAP also coincided with the accumulation of actin at

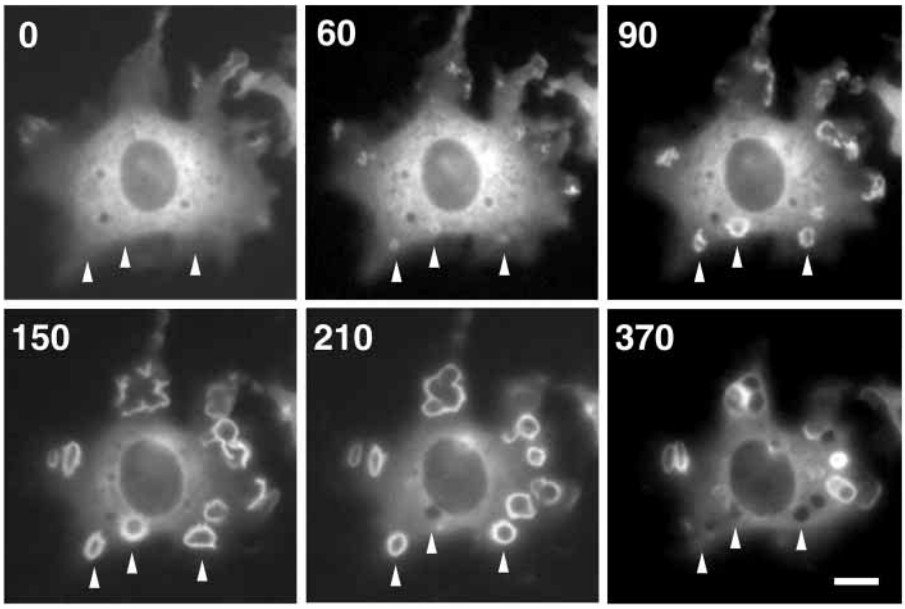

Fig. 3. Dynamics of GFP-VASP during early events of phagocytosis. Raw264.7 macrophages expressing GFP-VASP were incubated with IgG-opsonised sheep RBC and followed by digital video microscopy. GFP-VASP begins to accumulate at the sites of engulfment approximately one minute after SRBC binding, remains associated with phagocytic cups during until the entire internalisation process and disappears upon closure of the phagocytic cups. The positions of bound sheep RBC were observed using DIC optics (not shown). Arrowheads indicate sites of sheep RBC binding that led to complete phagosome formation. Numbers represent elapsed time (in seconds) after sheep RBC settled onto cells. Scale bar: 10 μm.

the phagocytic cups (Fig. 2D) and paralleled, in time and space, the localisation of VASP to the same structures. These observations fully agree with the data we obtained using Raw264.7 cells and support the notion that Ena/VASP proteins and Fyb/SLAP are involved in the actin-based processes underlying phagocytosis.

Recruitment of Ena/VASP proteins and Fyb/SLAP to nascent phagosomes is transient, occurring early during phagocytosis

To examine the temporal dynamics of Ena/VASP accumulation during phagosome formation, we used a GFP-tagged VASP construct for transfection into Raw 264.7 cells. Fig. 3 shows a series of time-lapse images of GFP-VASP-expressing Raw 264.7 macrophages exposed to IgG-opsonised sheep RBC. GFP-VASP is mostly cytoplasmic in resting cells (Fig. 3) (0 seconds), closely resembling the distribution of endogenous VASP (not shown). As phagocytosis proceeds, GFP-VASP accumulates at the sites of sheep RBC interaction within one minute of SRBC binding (Fig. 3) (60 seconds, see arrowheads). By 90 seconds, phagosomal cups enriched in GFP-VASP can be seen. The accumulation of GFP-VASP persists as phagosome formation proceeds, paralleling the recruitment of F-actin described previously (Furukawa and Fechheimer, 1994; Maniak et al., 1995), and then decreases as phagosome formation is completed (Fig. 3) (370 seconds). Thus, like F-actin accumulation, enrichment of Ena/VASP at forming phagosomes is transient and occurs early in the phagocytic process, specifically during particle engulfment.

In light of the above findings, we then analysed the dynamics of Fyb/SLAP accumulation during phagosome formation in GFP-Fyb/SLAP-expressing Raw 264.7 macrophages. Fig. 4 shows a series of time-lapse images of a Raw264.7 macrophage transfected with GFP-Fyb/SLAP and exposed to IgG-opsonised sheep RBC. GFP-Fyb/SLAP is mostly cytoplasmic in resting cells (Fig. 4) (0 seconds), and it can be seen to accumulate at the sites of sheep RBC interaction within approximately 90 seconds (Fig. 4). The accumulation of GFP-Fyb/SLAP persists throughout the process of particle engulfment, but tapers off and eventually dissipates by the time phagosome formation is complete (Fig. 4) (390 seconds). GFP-Fyb/SLAP was never found in association with sealed,

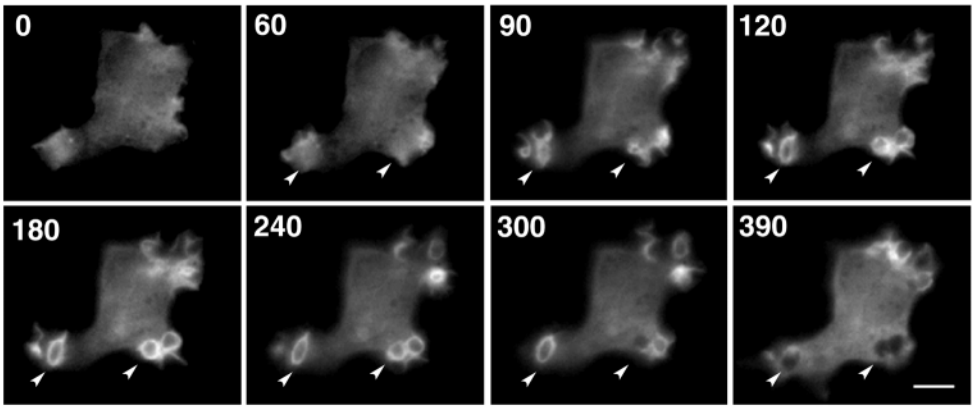

Fig. 4. Dynamics of GFP-Fyb/SLAP during early events of phagocytosis. Raw264.7 macrophages expressing GFP-Fyb/SLAP were incubated with IgG-opsonised SRBC and followed by video microscopy. GFP-Fyb/SLAP begins to accumulate at the sites of engulfment approximately one minute after sheep RBC binding, remains associated with phagocytic cups during the entire internalisation process and disappears upon closure of the phagocytic cups. The positions of bound sheep RBC were observed using DIC optics (not shown). Arrowheads indicate sites of sheep RBC binding that led to complete phagosome formation. Numbers represent elapsed time (in seconds) after SRBC settled onto cells. Scale bar: 10 μm.

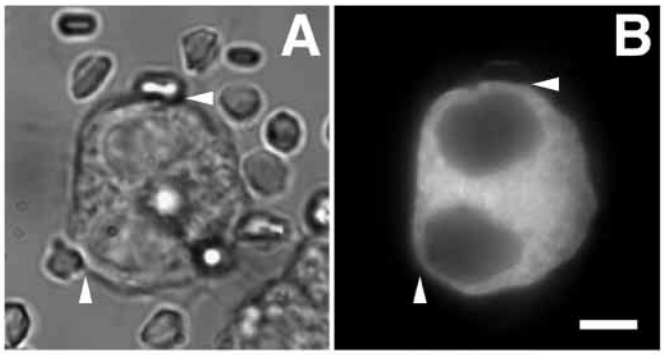

Fig. 5. *Clostridium difficile* toxin B inhibits the recruitment of VASP. Raw264.7 macrophages expressing GFP-VASP were incubated with 10 ng/ml *C. difficile* toxin B for two hours prior to use in phagocytosis assays with IgG-opsonised sheep RBC. Toxin B-treated cells bind sheep RBCs but do not internalise them (arrowheads in A). Note that there was no accumulation of GFP-VASP on the membrane abutting the bound particles (arrowheads in B). Scale bar: 10μm.

maturing phagosomes. A similar course of association and subsequent dissociation was recorded in more than 50 cells. This pattern was virtually identical to that reported above for GFP-VASP.

Recruitment of Ena/VASP family proteins during phagocytosis is dependent upon members of the Rho family of GTPases

Recent work has implicated members of the Rho subfamily of GTPases in regulating the actin remodelling that supports phagocytosis (Hackam et al., 1997; Caron and Hall, 1998). It is therefore possible that the recruitment of Ena/VASP proteins at sites of phagocytosis is dependent upon the activity of these GTPases. To test this possibility, Raw264.7 macrophages were transfected with GFP-VASP and then treated with Clostridium difficile toxin B prior to analysis. C. difficile toxin B is a bacterial toxin that can enter the cytosol of a variety of cells where it glucosylates and thereby inactivates Rho proteins (Just et al., 1995). Following treatment with toxin B, Raw264.7 cells bound IgG-opsonised RBC to their surface, but were unable to internalise them (Fig. 5A) in accordance with the reported requirement for functional Rho GTPases in phagocytosis. Furthermore, there was no discernible accumulation of GFP-VASP at sites of particle binding in toxin-treated cells (Fig. 5B). These findings imply that Rho-type GTPases are necessary for recruitment and/or stabilisation of Ena/VASP at the phagocytic cup.

Ena/VASP family proteins are essential for the FcγR receptor-mediated remodelling of the actin cytoskeleton

The observation that Fyb/SLAP and Ena/VASP proteins localise to nascent phagosomes prompted us to determine whether the recruitment of Ena/VASP proteins at sites of phagocytosis is imperative for the remodelling of the actin cytoskeleton. For this purpose, we used the approach recently developed by Krause et al. (Krause et al., 2000). We transfected Raw264.7 cells with GFP-tagged ActA repeats and analysed the effect of displacing Ena/VASP proteins from their endogenous ligands on FcγR-mediated actin cytoskeleton rearrangement. As a control, a mutated, inactive variant of the ActA repeats was expressed in these cells (Krause et al., 2000). Raw264.7 cells expressing the GFP-ActA repeats were still able to interact with opsonised erythrocytes (Fig. 6A-A″). However, both the accumulation of F-actin and the formation of the actin cups were inhibited, indicating that the remodelling of the actin cytoskeleton was impaired (Fig. 6A-A′ upper arrows). The inhibition of phagocytosis was exclusively due to the displacement of Ena/VASP proteins, as indicated by the observation that the GFP-ActA repeats displaced Evl from the sites where sheep RBC were bound to Raw264.7 cells (not shown). Conversely, in control untransfected cells and Raw264.7 cells expressing the mutated, inactive form of the ActA repeats, F-actin accumulated normally to form actin cups at the site of interaction between macrophages and sheep RBC (Fig. 6B-B″).

A quantification of the phagocytic efficiency showed that the number of internalised sheep RBC was greatly reduced in cells transfected with the GFP-ActA repeats (n=35) compared to

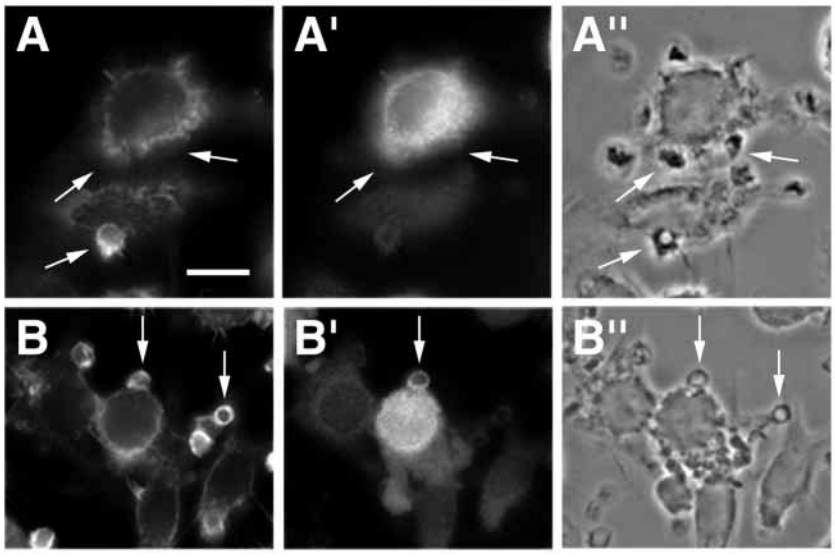

Fig. 6. Inhibition of actin cup formation by ActA repeats. Raw264.7 cells transiently transfected with GFP-tagged ActA repeats (A-aA″) or with the mutated form of the repeats (B-B″) were incubated with antibody-coated sheep RBC, fixed and stained with Texas Red-conjugated phalloidin. Cells expressing the active form of the ActA repeats were able to bind sheep RBC but did not form actin cups (arrows in A, A″). In contrast, the formation of actin cups was not affected in control (untransfected) cells and in cells that expressed the mutated form of the ActA repeats (arrows in B, B″). A, B: phalloidin lebelling; A′, B′: GFP signal; A″, B″: phase contrast. Scale bar: 10 μm.

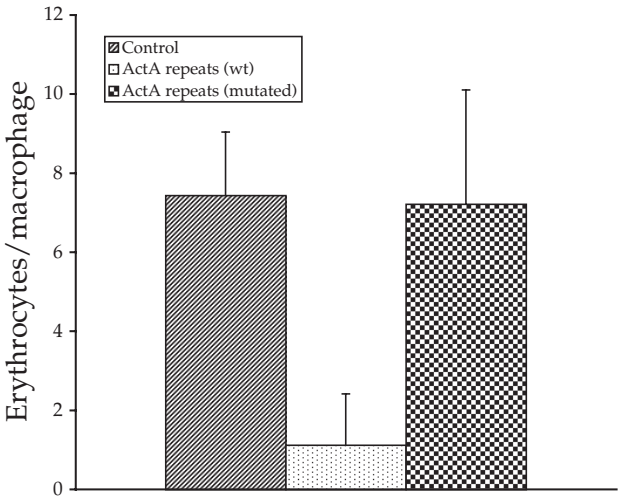

Fig. 7. Influence of ActA repeats on the rate of phagocytosis. Raw264.7 cells were transiently transfected with wild-type or mutated GFP-tagged ActA repeats and then incubated with antibody-coated sheep RBC. After incubation at 37°C for 15 minutes, non-internalised sheep RBC were lysed with distilled water and the cells fixed. The phagocytic efficiency is expressed as the number of internalised sheep RBC per macrophage. Error bars indicate one standard deviation from the mean.

untransfected cells (n=35) or cells expressing the inactive variant of the ActA repeats (n=19) (Fig. 7).

To investigate whether or not the displacement of Ena/VASP proteins was accompanied by discernible morphological changes, we analysed the phagocytic process by scanning electron microscopy. In Raw264.7 cells expressing GFP-ActA repeats, we found that, although sheep RBC attached to the plasma membrane, no lamellipodia-like extensions were formed (Fig. 8B). In untransfected macrophages or cells expressing the mutated form of the ActA repeats, the sheep RBC were almost completely surrounded by pseudopodial extensions (Fig. 8A,C) that eventually engulfed them completely. Thus, we conclude that proteins of the Ena/VASP family are essential for the FcγR-mediated remodelling of the actin cytoskeleton.

SLP-76, Nck and Vav localise to nascent phagocytic cups

Engagement of the T cell receptor induces the activation of several protein tyrosine kinases which, in turn, phosphorylate many adapter proteins of the T cell signal transduction pathway (Weiss and Littman, 1994; Clements et al., 1999). One of these proteins is SLP-76 (Jackman et al., 1995), which, upon phosphorylation, interacts with both Fyb/SLAP (Da Silva et al., 1997; Musci et al., 1997) and Vav (Tuosto et al., 1996), a guanine nucleotide exchange factor (GEF) for Rho GTPases (Olson et al., 1996; Crespo et al., 1997; Han et al., 1997). Moreover, T cell activation also leads to the interaction of SLP-76 with Nck (Bubeck Wardenburg et al., 1998).

On the basis of these observations and our finding that Fyb/SLAP localises to phagosomal cups, we reasoned that SLP-76, Nck and Vav may also be involved in the phagocytic process. To address this question, we examined the distribution of these proteins during phagocytosis in Raw264.7

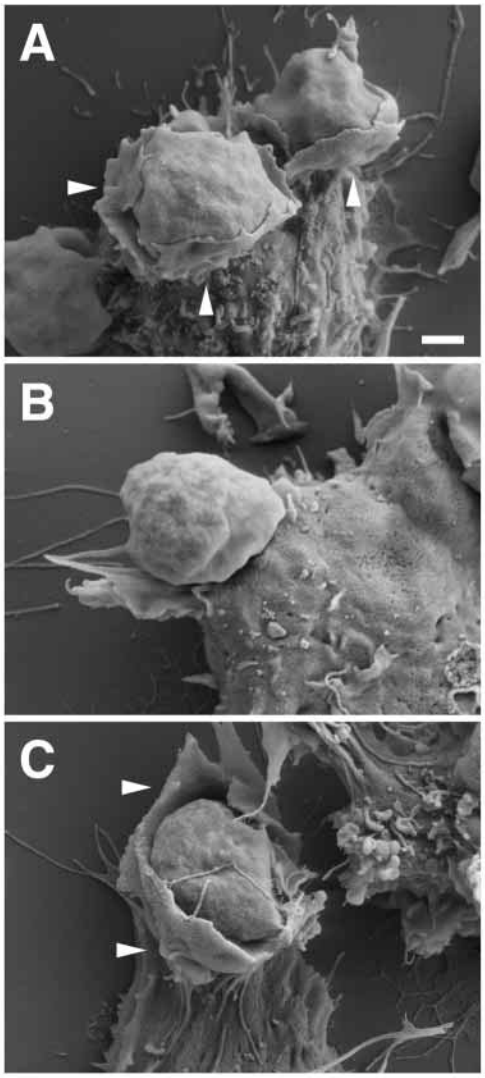

Fig. 8. Scanning electron microscopy analysis of the interaction between Raw264.7 cells and sheep erythrocytes. Untransfected Raw cells (A), cells expressing GFP-tagged ActA repeats (B), or the mutated GFP-tagged ActA repeats (C) were incubated with opsonised sheep RBC, fixed and processed for scanning electron microscopy. Raw cells expressing GFP-tagged ActA repeats were still able to bind to SRBC but did not form lamellipodia-like extentions, which, in contrast, are formed both by control cells and cells expressing the inactive form of the ActA repeats. Scale bar: 2 μm.

macrophages ingesting IgG-opsonised sheep RBC. Within five minutes of the addition of opsonised sheep RBC, Nck (Fig. 9A′), Vav (Fig. 9B′) and SLP-76 (Fig. 9C′) accumulated at nascent phagosomal cups. All of these proteins colocalised with actin at the same sites (Fig. 9A-C). Thus, Nck, SLP-76 and Vav are involved in early events of phagocytosis and may link the FcR signalling pathway to the actin cytoskeleton.

Fyb/SLAP, SLP-76, Nck and WASP form a complex in stimulated Raw264.7 macrophages

Proteins of the WASP family have recently attracted much attention as regulators of the actin cytoskeleton (Sechi and Wehland, 2000). WASP is essential for T cell function and

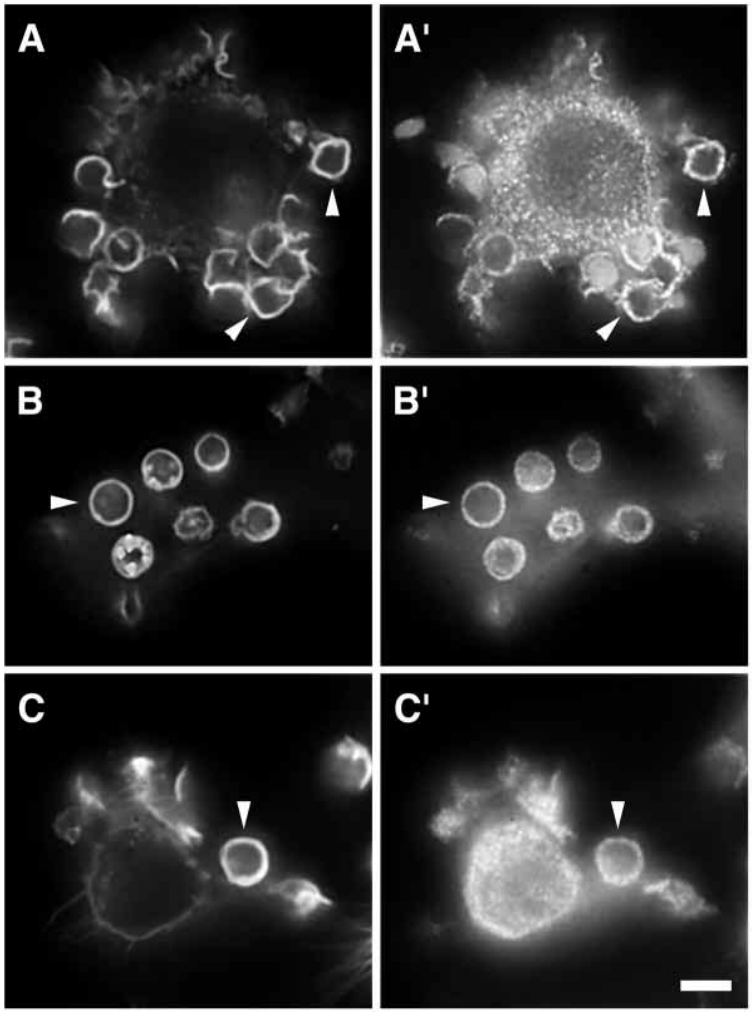

Fig. 9. Localisation of signalling proteins to phagocytic cups. Raw264.7 cells were incubated with antibody-coated sheep RBC, fixed and co-stained with Texas Red-conjugated phalloidin (A, C) and with antibodies against Nck (A′), Vav (B′) or SLP-76 (C′). The three signalling proteins all localise to the phagocytic cups (arrowheads in A', C') where they colocalise with actin (arrowheads in A, C). Scale bar: 5 μm.

localises to the interface between T cells and anti-CD3-coated beads (Derry et al., 1994; Krause et al., 2000). Because of the similarity between T-cell receptor and FcR signalling pathways, we sought to determine whether WASP is involved in the formation of phagocytic cups. First, we analysed Raw264.7 cell lysates with an anti-WASP monoclonal antibody. As a positive control, we prepared cell lysates from Jurkat T-cells, which are known to express this protein (Krause et al., 2000). This monoclonal antibody detected a single prominent band of approximately 65 kDa, the predicted molecular mass of WASP, indicating that this protein is expressed in Raw264.7 macrophages (Fig. 10A). Next, we analysed the distribution of WASP during phagocytosis. Immunolabelling of WASP was perfomed with a rabbit polyclonal antibody that gave a higher signal-to-noise ratio than the anti-WASP monoclonal antibody. This polyclonal antibody also recognised a single band of 65kDa in lysates of Raw264.7 cells (not shown). To exclude crossreaction with the polyclonal antibody we used to opsonise sheep RBC, we studied phagocytosis using IgG-opsonised beads. Within five minutes of the addition of the beads, WASP accumulated at forming phagosomes (Fig. 10B, right panel) where it colocalised with F-actin (Fig. 10B, left panel). The spatial and temporal accumulation of WASP at nascent phagocytic cups was similar to the accumulation of the other proteins we had analysed so far. These observations are consistent with the findings of Lorenzi et al. (Lorenzi et al., 2000), who showed that the phagocytosis is inhibited in macrophages lacking WASP and indicate that WASP is essential for the actin-based process accompanying phagocytosis.

As Fyb/SLAP, Ena/VASP proteins, Nck, Vav, SLP-76 and WASP localised to the phagosomal cups in Raw 264.7 cells, we speculated that these proteins might participate in the formation of a multiprotein complex during phagocytosis. This possibility is consistent with observations indicating that Fyb/SLAP interacts with SLP-76 (Da Silva et al., 1997; Musci et al., 1997) which, in turn, forms a complex with Vav and Nck (Bubeck Wardenburg et al., 1998). On the other hand, WASP binds to the SH3 domain of Nck (Rivero-Lezcano et al., 1995). Moreover, we recently demonstrated that this group of proteins forms a complex that is essential for the actin cytoskeleton remodelling in Jurkat T-cells (Krause et al., 2000).

To test this hypothesis, we prepared immunoprecipitates from extracts of control Raw264.7 and cells stimulated by Fcγ receptor crosslinking using the WASP monoclonal antibody. WASP immunoprecipitates were analysed using specific antibodies to Fyb/SLAP, SLP-76, Evl and Nck. Except for SLP-76, none of these proteins were detectable in WASP immunoprecipitates of control Raw264.7 cells (Fig. 10C). The small amount of SLP-76 recovered in these immunoprecipitates may be due to the formation of a complex between SLP-76 and Grb2, which is also able to interact with WASP (Jackman et al., 1995; She et al., 1997; Zhu et al., 1997). Conversely, all four of these proteins were found in WASP immunoprecipitates obtained from stimulated Raw264.7 cells, indicating that they are present in a multimolecular complex (Fig. 10C). In each of these blots control probes immunoprecipitated using immobilised myeloma IgG were negative.

To rule out unspecific trapping, we probed the same immunoprecipitates with a monoclonal antibody to zyxin, which is not detectable in phagosomal cups. As expected, zyxin could not be immunoprecipitated with WASP (not shown), proving the specificity of our approach.

Thus, these results are consistent with our previous investigations and support the notion that a multimolecular complex is required for FcRγ-mediated remodelling of the actin cytoskeleton.

DISCUSSION

It has long been appreciated that reorganisation of actin around forming phagosomes is an integral part of particle engulfment, but it is not understood how this modulation of actin is spatially

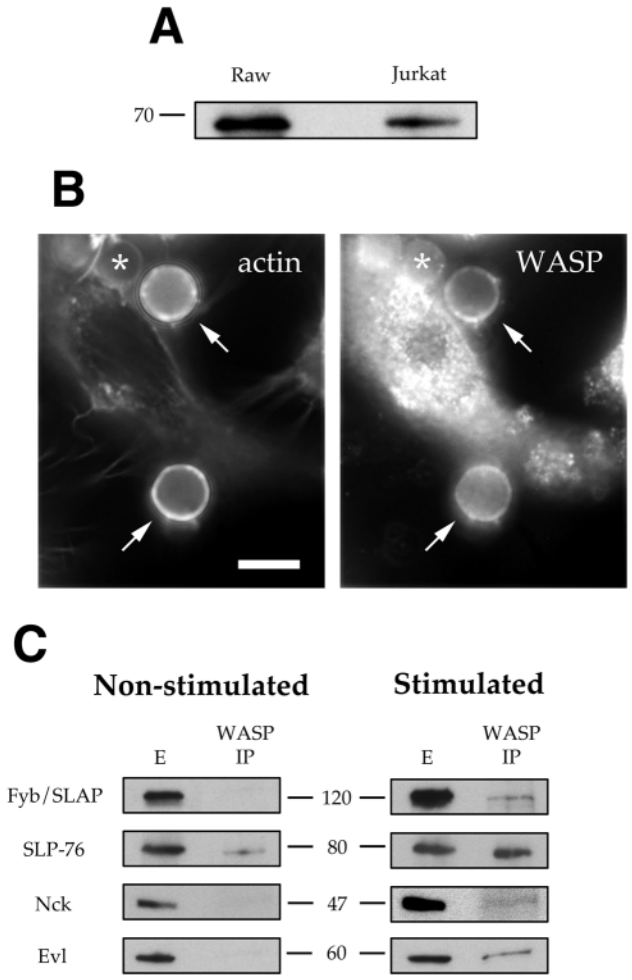

Fig. 10. *(*A) Analysis by western blotting of protein extracts of monocyte-derived macrophages and Jurkat T-cells with an anti-WASP monoclonal antibody. (B) Localisation of WASP in Raw264.7 cells undergoing phagocytosis. In macrophages engulfing IgG-opsonised beads, WASP colocalises with actin at phagocytic cups. Arrowheads point to sites where opsonised beads induced the formation of phagocytic cups. Stars indicate opsonised beads that have bound to a cell but have not induced the accumulation of actin or WASP. Scale bar: 10 μm. (C) Fyb/SLAP, SLP-76, Nck, Ena/VASP proteins and WASP are located in a multi-protein complex in stimulated Raw264.7 cells. Cell lysates from control and stimulated Raw264.7 cells were incubated with the immobilised WASP monoclonal antibody 67B4. Immunoprecipitates were resolved by SDS-PAGE, blotted and probed with antibodies to Fyb/SLAP, SLP-76, Nck and Evl. The stimulation of Raw264.7 cells results in the association of Fyb/SLAP, SLP-76, Nck and Evl with WASP. E, full Raw264.7 cell lysate; WASP IP, WASP immunoprecipitate.

and temporally orchestrated. In the present study, we demonstrate that Ena/VASP proteins are transiently recruited to sites of particle attachment during early stages of phagocytosis and that they are essential for an efficient phagocytic process. The recruitment of Ena/VASP proteins coincides with the accumulation of Fyb/SLAP, SLP-76, Nck, Vav and WASP. We also provide evidence that a multimolecular complex formed by Ena/VASP proteins, Fyb/SLAP, SLP-76, Nck and WASP, links the actin cytoskeleton to Fcγ-mediated signalling pathway during phagocytosis.

The EVH1 domains of Ena/VASP family proteins are thought to mediate the localisation of these proteins to sites of actin cytoskeleton remodelling (Chakraborty et al., 1995; Gertler et al., 1996; Carl et al., 1999; Laurent et al., 1999). EVH1-binding motifs have been found in cytoskeletal proteins such as zyxin and vinculin as well as in the *Listeria* protein ActA (Brindle et al., 1996; Reinhard et al., 1996; Niebuhr et al., 1997). We have recently demonstrated that Fyb/SLAP binds to the EVH1 domains of Ena/VASP proteins and that it colocalises with these proteins at the interface between T cells and anti-CD3-coated beads (Krause et al., 2000). Thus, these findings and this study suggest that, at least in haematopoietic cells, Fyb/SLAP is responsible for recruiting Ena/VASP proteins to sites of actin cytoskeleton remodelling. However, we cannot exclude the possibility that other as yet unidentified Ena/VASP cognate ligands are involved in their targeting. Alternatively, vinculin, which localises to phagocytic cups (Allen and Aderem, 1996a), might participate in the targeting Ena/VASP proteins to these sites. We, however, could not detect vinculin at nascent phagosomes (data not shown).

Presently, it is not known whether there are one or more mechanisms directing the targeting of Ena/VASP proteins to highly dynamic subcellular regions. In this context, Bear et al. (Bear et al., 2000) have recently shown that in fibroblasts the targeting of Ena/VASP proteins to dynamic cellular regions (e.g. lamellipodia) might be independent from their interaction with EVH1-binding motifs. If this were also true for the phagocytic process, we would have expected no or little inhibition of phagocytosis in macrophages expressing the GFP-ActA repeats. Thus, we hypothesise that the EVH1-mediated interaction between Fyb/SLAP and Ena/VASP proteins plays an essential role in phagocytosis. In addition, experiments using *C. difficile* toxin B indicated that the activity of Rho subfamily members is required for the localization of VASP to sites of phagosome formation (Fig. 5). This finding can explain, at least in part, previous observations documenting the requirement for Rho-related proteins in phagocytosis (Hackam et al., 1997; Caron and Hall, 1998). The precise mechanism whereby Rho-related GTPases regulate VASP localization remains to be defined, but it is reasonable to speculate that their activity is required for early steps in the assembly of the molecular complex to which VASP is recruited, probably including the protein(s) containing the critical EVH1-binding motifs.

When the proper localisation of Ena/VASP proteins is perturbed, a deficit in actin filaments near the attached particle may prevent proper membrane remodelling and extension, impairing phagosome formation. One explanation for the effects of displacing Ena/VASP proteins from sites of phagocytosis is that a lack of these proteins inhibits the recruitment of profilin, an actin-monomer-binding protein that is a ligand for Ena/VASP proteins. In this context, we observed that, although its accumulation was not dramatic, GFP-tagged profilin localised at the sites of phagocytosis (not shown). Thus, if the Ena/VASP-mediated recruitment of profilin to forming phagosomes is impaired, then the resulting supply of available actin monomers may be insufficient and may therefore impede filament formation. This notion is supported by the observation that ActA-repeat-induced delocalisation of

Ena/VASP proteins impairs the accumulation of F-actin at the sites of sheep RBC attachment.

T-cell-receptor- and Fcγ-receptor-mediated signalling pathways have similar downstream components. Both T cell receptor and Fcγ receptor engagement cause the stimulation of protein tyrosine kinases that leads to the phosphorylation of the ITAMs of these receptors (Greenberg et al., 1993; Greenberg et al., 1994; Weiss and Littman, 1994; Clements et al., 1999). Among other downstream proteins that become tyrosine-phosphorylated upon activation of these signalling pathways are LAT (Tridandapani et al., 2000; Zhang et al., 1998) and SLP-76 (Jackman et al., 1995; Bonilla et al., 2000). Fyb/SLAP interacts with SLP-76 upon T cell receptor activation (Da Silva et al., 1997). Thus, because of this interaction, it is possible that Fyb/SLAP (and also Ena/VASP proteins) is recruited to the forming phagosome through its direct interaction with SLP-76. Another consequence of T cell receptor clustering is the formation of a ternary complex including SLP-76, Vav and Nck (Bubeck Wardenburg et al., 1998). Our observation that these three proteins localise to nascent phagosomes suggests that they are also involved in the Fcγ receptor signalling pathway.

We have recently shown that, after T cell receptor stimulation, Fyb/SLAP and WASP colocalise at the interface between T cells and anti-CD3-coated beads and that a complex containing these proteins can be immunoprecipitated with WASP (Krause et al., 2000). Moreover, in agreement with the observation that the Arp2/3 complex is required for efficient phagocytosis (May et al., 2000), we found that the Arp2/3 complex co-immunoprecipitates with WASP (not shown). In light of the present study, it is tenable that similar molecular interactions are connecting receptor function to actin reorganisation in both T cells and macrophages. Our current observations support the conclusion that signalling between the Fcγ receptor and the actin cytoskeleton is mediated by a multimolecular complex containing SLP-76, Fyb/SLAP, Ena/VASP proteins, Nck and WASP. In this complex, SLP-76 may be a central component, binding both Fyb/SLAP, which then recruits Ena/VASP proteins and profilin, and Nck, which in turn recruits WASP and Arp2/3. Thus, during phagocytosis SLP-76 may partake in interactions that form two axes of a molecular network, one connected to the regulation of the availability of actin monomers and one connected to the nucleation of actin filaments. Together these axes would permit spatially and temporally controlled actin remodelling, directly promoting phagosome formation. The formation of these two molecular axes seems to be independent, as suggested by our finding that the displacement of Ena/VASP proteins does not affect the localisation of WASP at phagocytic sites (data not shown) or T-cell/beads interfaces (Krause et al., 2000).

Our hypothesis does not exclude the possibility that other hitherto unidentified components also participate in the targeting of the actin polymerisation machinery to clustered Fcγ receptors. For example, one such component may be the B cell linker protein (BLNK). Also known as SLP-65, BLNK is involved in Fcγ-receptor-mediated signalling and supports the phagocytosis of sheep RBC in SLP-76$^{-/-}$ macrophages, suggesting a functional similarity to SLP-76 (Bonilla et al., 2000).

Overall, our present observations are consistent with a model of actin rearrangement that requires the coupling of the activities of Ena/VASP family members and the Arp2/3 complex. Along with T-cell-receptor-triggered actin reorganisation, this model is similar to that of the actin polymerisation that is regulated by Ena/VASP proteins and the Arp2/3 complex during the actin tail formation that accompanies *Listeria* motility (Niebuhr et al., 1997; Welch et al., 1997a; Laurent et al., 1999; May et al., 1999). It is increasingly apparent that molecular interactions that link Ena/VASP proteins to the Arp2/3 complex are a common theme in the regulatory mechanisms of many actin-cytoskeleton-dependent cellular processes. Furthermore, in several systems the molecules involved appear to be quite similar. This improved understanding of the signalling pathways that link immune receptor functions to dynamic actin structures may prove to be an important resource in the quest for treatments of both disorders of the immune system and infectious diseases.

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