Annexin A1 is a new functional linker between actin filaments and phagosomes during phagocytosis

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Summary

Remodelling of the actin cytoskeleton plays a key role in particle internalisation and the phagosome maturation processes. Actin-binding proteins (ABPs) are the main players in actin remodelling but the precise role of these proteins in phagocytosis needs to be clarified. Annexins, a group of ABPs, are known to be present on phagosomes. Here, we identified annexin A1 as a factor that binds to isolated latex bead phagosomes (LBPs) in the presence of Ca^{2+} and facilitates the F-actin–LBP interaction in vitro. In macrophages the association of endogenous annexin A1 with LBP membranes was strongly correlated with the spatial and temporal accumulation of F-actin at the LBP. Annexin A1 was found on phagocytic cups and around early phagosomes, where the F-actin was prominently concentrated. After uptake was completed, annexin A1, along with F-actin, dissociated from the nascent LBP surface. At later stages of phagocytosis annexin A1 transiently concentrated only around those LBPs that showed transient F-actin accumulation ('actin flashing'). Downregulation of annexin A1 expression resulted in impaired phagocytosis and actin flashing. These data identify annexin A1 as an important component of phagocytosis that appears to link actin accumulation to different steps of phagosome formation.

Key words: Annexin A1, F-actin, LBP, Phagocytosis

Introduction

Cells use different strategies to internalise particles and solutes, including pinocytosis, receptor-mediated endocytosis and phagocytosis. Phagocytosis is a universal cell function that exploits a ubiquitous and mostly conserved cell machinery to couple receptor-dependent binding of particulate material (>0.5 μm in diameter) to its internalisation. Although primitive organisms use phagocytosis primarily for acquisition of nutrients, phagocytosis in metazoans occurs in specialised phagocytic cells, such as macrophages, dendritic cells and neutrophils. (Aderem, 2002; Aderem and Underhill, 1999; Castellano et al., 2001; Greenberg and Grinstein, 2002; Underhill and Ozinsky, 2002). The molecular mechanisms underlying phagocytosis are extremely complex and not precisely defined, but recent studies have described many aspects of the process. Phagocytosis is initiated by binding of specific ligands on the particles to their cognate receptors, such as Fcγ, mannose and complement receptors, which trigger intracellular signals. These signalling cascades lead to the polymerisation and rearrangement of filamentous actin (F-actin) beneath the particle and coordinate the tractional forces that internalise the particles (Aderem and Underhill, 1999; Castellano et al., 2001). Different receptors generate different signalling cascades, which have distinct effects on the actin cytoskeleton, and different biological responses (Allen and Aderem, 1996; Caron and Hall, 1998; Kuiper et al., 2008). For example, Fcγ receptor (FcγR)-mediated phagocytosis requires the Cdc42-Rac-Rho signalling pathway to modify the actin cytoskeleton, whereas complement receptor only requires Rho GTPase activity for the F-actin rearrangement (Caron and Hall, 1998).

With the help of the actin cytoskeleton, particles get engulfed and form the phagosomes, which harbour a number of characterised and uncharacterised polypeptides, including phagocytic receptors, cytoskeleton proteins [e.g. actin and actin-binding proteins (ABPs)], signalling molecules (e.g. protein kinase C) and membrane trafficking proteins (e.g. Rab 5 and Rab 7). As for the signalling events, different receptors influence the protein composition of phagosomes (Hoffmann et al., 2010). In addition, with ongoing maturation, the protein and lipid composition of the phagosome alters (Desjardins et al., 1994a; Haas, 2007). In the past decade, several proteomic studies have set out to determine the protein composition of phagosomes (Desjardins et al., 1994a; Hoffmann et al., 2010; Griffiths and Mayorga, 2007; Martinez-Solano et al., 2006; Morrissette et al., 1999; Slomianny et al., 2006), but most of these studies did not focus on the individual functions of these proteins. Like many other proteins, ABPs are known to be present on the phagosomes, but their role in the biogenesis of phagosomes is still poorly understood.

Among the ABPs present on phagosomes are proteins of the annexin family (Diakonova et al., 1997). Annexins are type II (non-EF hand) Ca^{2+}-binding proteins, which bind to negatively charged phospholipids in the presence of Ca^{2+}. Annexins comprise four or eight 70-amino-acid repeats and a variable N-terminus, which is believed to be responsible for their different activities (Moss, 1992). Previous studies suggested that annexins participate in a broad range of intracellular processes, including membrane dynamics, membrane cytoskeleton interactions and vesicle trafficking (Futter and White, 2007; Gerke et al., 2005; Moss, 1992).

Annexin A1, formerly known as lipocortin 1, was initially identified as an anti-inflammatory protein that is glucocorticoid regulated and secreted atypically from cells (D'Acquisto et al., 2008). Intracellularly, annexin A1 is predominantly a cytosolic

protein, but it is also found on the plasma membrane and cellular organelles, such as endosomes, phagosomes and multivesicular bodies, where the protein participates in inward vesiculation (Futter and White, 2007; Gerke et al., 2005; Lim and Pervaiz, 2007). The presence of annexin A1 on the phagosomal membrane seems to be functionally important in macrophages and neutrophils. Rapid translocation of annexin A1 to the phagosome membrane has been observed during the phagocytosis of nonpathogenic or killed bacteria (Harricane et al., 1996; Kaufman et al., 1996), and macrophages from annexin-A1-knockout mice showed impaired phagocytosis of non-opsonised zymosan (Yona et al., 2005). In addition, knockout of annexin A1 has been shown to alter cytokine production and phagocytic receptor expression in mouse macrophages (Yang et al., 2006; Yang et al., 2009; Yona et al., 2004; Yona et al., 2006). Although the mechanistic basis of the action of annexin A1 on phagosomes is not known, in vitro data suggest a link to the actin cytoskeleton. Annexin A1 binds to and bundles F-actin in vitro and colocalises with F-actin in different cell lines (Hayes et al., 2004). It can also interact with profilin and is believed to be involved in actin dynamics, at least in vitro (Alvarez-Martinez et al., 1996; Alvarez-Martinez et al., 1997).

We have demonstrated previously that at least two types of ABPs, ATP-dependent and ATP-independent ABPs, are involved in the phagosome and F-actin interaction, and have identified one of the ATP-dependent ABPs as myosin Va (Al Haddad et al., 2001). However, in that study another stimulatory ATP-independent factor was not identified. Here, we found that annexin A1 facilitates the phagosome and F-actin interaction in an ATP-independent manner in vitro. We also found that annexin A1 colocalised with F-actin during phagocytic cup formation and on mature phagosomes in vivo. The presence of annexin A1 on phagosomes in cells strongly correlated with the time-dependent association of F-actin with phagosomes. The decrease in phagosome-associated F-actin after phagocytic cup formation also correlated with a decrease in the levels of associated annexin A1. Furthermore, annexin-A1-knockdown resulted in a diminished phagocytic activity in mouse macrophages. Thus, annexin A1 appears to be involved in controlling association of F-actin with phagosomes, thereby affecting phagosome formation.

Results

Isolation of the cytosolic fraction with enriched F-actin–LBP binding activity

To study the phagosome–F-actin interaction, an in vitro actin-binding assay has been established in our laboratory using isolated latex bead phagosomes (LBPs) (Al Haddad et al., 2001). Here, we used this assay to look for new ABPs involved in the F-actin–LBP interaction. In order to achieve this goal, macrophage cytosol was fractionated by standard size-exclusion chromatography (gel filtration). The ensuing protein-containing fractions were analysed for their ability to stimulate F-actin–LBP binding. The majority of the activity was found in the initial fractions, which contain higher molecular mass proteins or protein complexes (Fig. 1A). SDS-PAGE analysis showed that these active fractions not only contained high molecular mass proteins, but also many proteins of low molecular mass (Fig. 1B). For further purification, the active fractions were pooled and subjected to cation-exchange chromatography on phosphocellulose resin (P11). Almost all of the activity was found to reside in the resin-bound fraction, whereas the resin-unbound fraction even caused inhibition in F-actin–LBP binding activity, probably owing to an enrichment of inhibitory

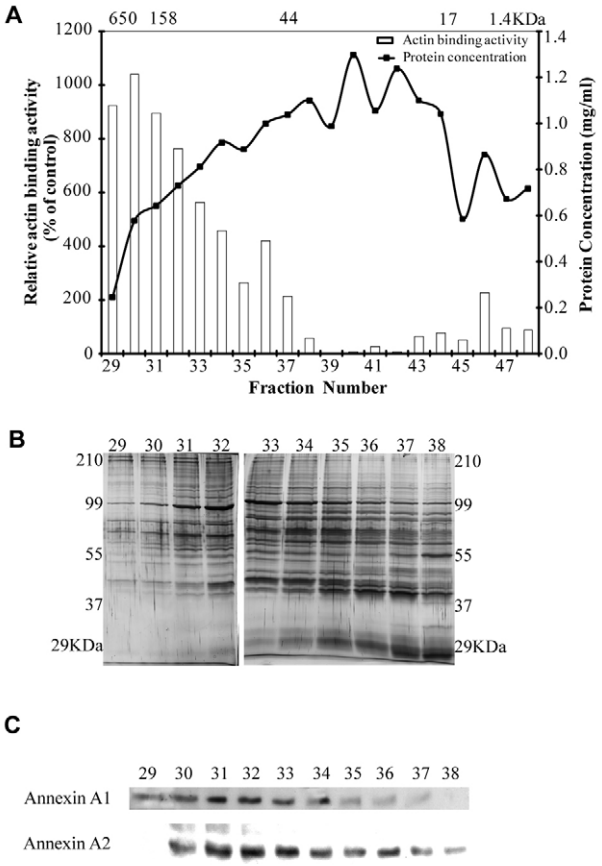

Fig. 1. Fractionation of cytosol from J774A.1 macrophages by gel filtration. (**A**) The protein concentrations and actin-binding activity of fractions obtained by gel filtration. The positions of molecular mass markers (kDa) are shown at the top. (**B**) SDS-PAGE analysis of the first ten protein-containing fractions. The positions of molecular mass markers are shown on the left- and right-hand sides. (**C**) Immunoblotting of the fractions with antibodies against annexin A1 or annexin A2.

factors (Fig. 2A). The specific F-actin–LBP binding activity found in proteins eluted from the phosphocellulose resin was approximately eight times higher than in cytosol and five times higher than in gel-filtration fractions (Table 1).

Proteomic analysis of phosphocellulose-eluted proteins

For further identification of the proteins responsible for the F-actin–LBP interaction, the phosphocellulose-eluted fraction was analysed by mass spectrometry (LC-MS/MS; liquid chromatography tandem mass spectrometry), which identified 179 proteins. Considering the high sensitivity of the method and amount of the protein used for identification, we decided to use very stringent criteria for filtering the results. Hence, as a first filter, protein hits with a less than 95% protein identification probability and without at least two unique peptides were removed; this resulted in 75 proteins of interest. Given that the protein(s) of interest should also have phagosome-binding properties, we compared our LC-MS/MS data with those previously obtained using mass spectrometry analysis of isolated LBPs by Desjardins and colleagues (Desjardins et al., 1994a) and Hoffmann and colleagues (Hoffmann et al., 2010). This comparison identified 22 proteins known to be present on phagosomes. In addition, the

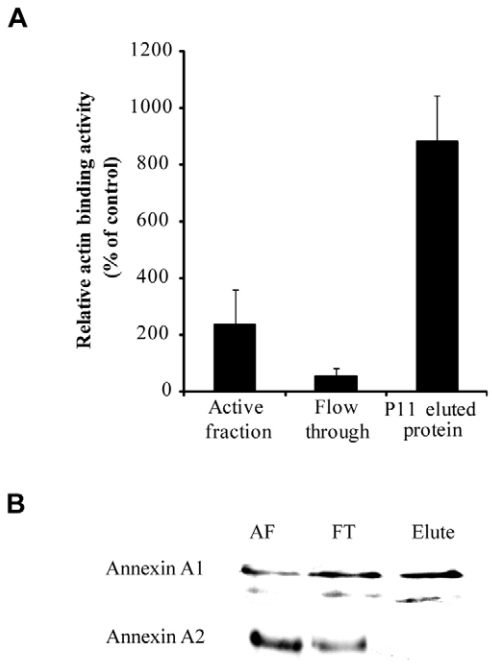

Fig. 2. Phosphocellulose chromatography of pooled fractions obtained by gel filtration. (**A**) Relative F-actin–LBP binding activity of loaded pooled (active fraction; AF) fractions, unbound (flowthrough; FT) and eluted proteins. The results are the means+s.d. for five independent experiments. (**B**) Immunoblotting of the phosphocellulose fractions with antibodies against annexin A1 or annexin A2.

protein(s) of interest have to be ABP(s), which can link the phagosomes and F-actin. According to a literature survey, only 11 of the 22 proteins had a known actin-binding property (Table 2). Out of these 11 proteins, only three, annexin A1, annexin A2 and moesin, have known lipid-binding properties and, thus, were considered for further investigation. Given that moesin, a member of the ERM protein family, is a known nucleator of actin filament assembly on the phagosome membrane, and cannot bind to the lateral surface of preformed F-actin networks (Bretscher et al., 2000; Defacque et al., 2000), it could not be the protein of interest. Hence, we reduced the list to the two most probable candidates: annexin A1 and annexin A2.

To confirm the LC-MS/MS data, immunoblotting of the protein fractions was performed after the gel-filtration and the cation-exchange chromatography. Both annexin A1 and annexin A2 were detected in the initial fractions after gel-filtration (Fig. 1C), but only annexin A1 was strongly enriched among the phosphocellulose-eluted proteins analysed by LC-MS/MS (Fig. 2B). Therefore, we concluded that annexin A1 might be the protein that is responsible for mediating the F-actin–LBP interaction we detected in vitro in the above experiment.

Recombinant annexin A1 stimulates F-actin–LBP interaction in a Ca^{2+}-dependent manner in vitro

To support our hypothesis that annexin A1, but not annexin A2, which is a close homologue in the annexin family, is responsible for the F-actin–LBP interactions, we performed an in vitro actin-binding assay in the presence of bacterially expressed recombinant full-length annexin A1 or annexin A2. As expected, both annexin A1 and annexin A2 failed to stimulate the F-actin–LBP interaction in the absence of Ca^{2+} (Fig. 3). However, in the presence of Ca^{2+}, annexin A1 strongly stimulated the F-actin–LBP interaction, whereas annexin A2 failed to show a significant effect (Fig. 3). Given that S100A10, the light chain of annexin A2, is important for several of its biological activities (Miwa et al., 2008), the effect of annexin A2 on the F-actin–LBP binding activity was also analysed using an annexin A2–S100A10 heterotetramer complex purified from pig intestine (Gerke and Weber, 1984). However, in a similar manner to bacterially expressed annexin A2, this heterotetramer complex also failed to stimulate the F-actin–LBP interaction, both in the presence and absence of Ca^{2+} (data not shown). These data support our hypothesis that annexin A1, but not annexin A2, is a protein that is responsible for mediating the F-actin–LBP interaction in vitro.

Table 1. Purification of cytosolic fractions with enriched F-actin–LBP binding activity

Purification step	Protein concentration (mg/ml)	LBPs per field	Specific activity (SLBP per field per mg of protein)	Enrichment of specific activity during purification (%)
Control (buffer)	–	3	–	–
Cytosol	0.50	17.1	34	100
Gel-filtration fraction	0.49	27.4	56	165
Cation chromatography	0.10	26.4	264	773

Table 2. Selected list of ABPs detected by LC-MS/MS in cytosolic fractions with enhanced F-actin–LBP binding activity

No.	Protein name and symbol	Accession number (International Protein Index)	Protein molecular mass (kDa)	Reference
1	Annexin A1 (Anxa1)	IPI00230395	38.7	(Blackwood and Ernst, 1990)
2	Annexin A2 (Anxa2)	IPI00468203	38.6	(Blackwood and Ernst, 1990)
3	Moesin (Msn)	IPI00110588	67.7	(Nakamura et al., 1999)
4	Elongation factor 2 (Eef2)	IPI00466069	95.3	(Bektas et al., 1994)
5	Elongation factor 1 alpha 1 (Eef1a1)	IPI00307837	50.7	(Ejiri, 2002)
6	Elongation factor 1 alpha 2 (Eef1a2)	IPI00119667	50.4	(Ejiri, 2002)
7	Myosin-9 (Myh9)	IPI00123181	226.3	(Knetsch et al., 1999)
8	Ttll3 actin-related protein 2/3 complex subunit 4 (Arpc4)	IPI00138691	19.6	
9	2900073G15Rik myosin light chain, regulatory B-like	IPI00109044	19.9	(Fujita et al., 1999)
10	AHNAK nucleoprotein isoform 1 (Ahnak)	IPI00553798	604.2	(Benaud et al., 2004)
11	Pyruvate kinase isozyme M2 (Pkm2)	IPI00407130	58.0	(Arnold et al., 1971)

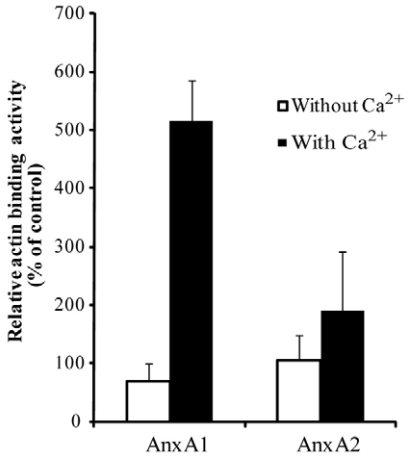

Fig. 3. In vitro actin-binding assay with purified exogenous annexin A1 or annexin A2. Bacterially expressed and purified annexin A1 (AnxA1) or annexin A2 (AnxA2) was incubated with the LBPs in the presence (With Ca^{2+}) or absence (Without Ca^{2+}) of Ca^{2+}. The results are the means+s.d. for five independent experiments.

Annexin A1 is present on isolated LBPs and binds to LBPs in a Ca^{2+}-dependent manner

The results described above have shown that exogenous bacterially expressed annexin A1 can facilitate the F-actin–LBP interaction. In order to confirm the role of exogenous annexin A1, its presence on isolated LBPs was analysed. As determined by immunofluorescence staining, annexin A1 was found on isolated LBPs (Fig. 4A) that were not treated with a high salt concentration (non-stripped). Isolated non-stripped LBPs have many membrane-associated proteins (Desjardins et al., 1994a; Hoffmann et al., 2010). When non-stripped LBPs were treated with 2 M NaCl, they lost nearly all F-actin-binding activity and a number of membrane-associated proteins (Al Haddad et al., 2001; Defacque et al., 2000). In line with these data, salt-stripping of LBPs also efficiently removed prebound annexin A1 from LBP membranes (Fig. 4B). Interestingly, the addition of bacterially expressed full-length annexin A1 to salt-stripped LBPs restored both the appearance of annexin A1 on LBPs and their F-actin-binding activity (Fig. 4C,D). Furthermore, this effect was strongly Ca^{2+} dependent (Fig. 4C). The fluorescence intensity of LBPs incubated with exogenous annexin A1 in the presence of Ca^{2+} was at least five times higher than that of LBPs incubated without Ca^{2+} (Fig. 4D,E).

Anti-(annexin A1) antibodies inhibit the F-actin–LBP interaction

Apart from being present on phagosomal membranes, annexin A1 was also found in cytosolic fractions that were enriched in F-actin–LBP binding activity (Fig. 2B). Therefore, we analysed the involvement of annexin A1 in the activity of these fractions by preincubating the annexin-A1-containing phosphocellulose-eluted fraction with rabbit polyclonal anti-(annexin A1) antibodies. As a control, rabbit polyclonal anti-moesin antibodies were used. Blocking of annexin A1 with the anti-(annexin A1) antibodies strongly diminished the F-actin–LBP interaction triggered by the phosphocellulose-eluted protein fraction (Fig. 5). By contrast, the anti-moesin antibodies did not have any significant effect on the F-actin–LBP interaction (Fig. 5). These data clearly argue that annexin A1 is the cytosolic protein from the phosphocellulose-eluted fraction that stimulates the F-actin–LBP interaction in vitro.

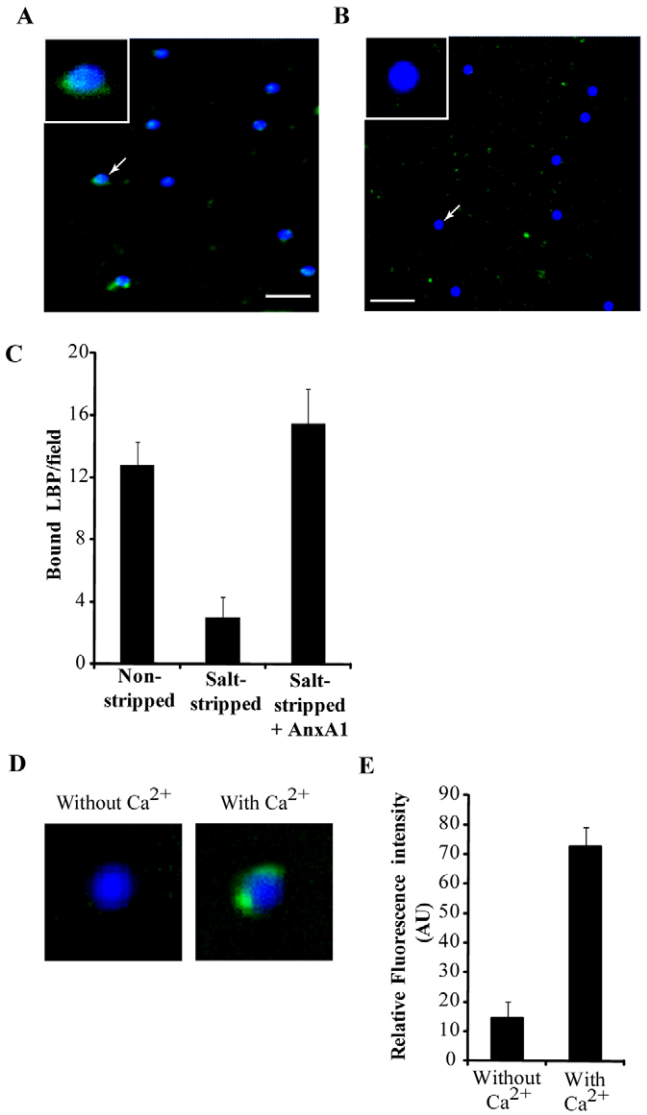

Fig. 4. Presence of annexin A1 on LBPs. LBPs (blue) were subjected to indirect immunofluorescence microscopy using an antibody against annexin A1 (green). (**A**) Non-stripped LBPs. (**B**) Salt-stripped LBP. Insets: the LBP marked with an arrow at 3× magnification. Scale bars: 5 µm. (**C**) Actin-binding activity of non-stripped LBPs (Non-stripped), salt-stripped LBPs (salt-stripped) and salt-stripped LBPs incubated with annexin A1 in the presence of Ca^{2+} (Salt-stripped + AnxA1). (**D**) Immunofluorescence of salt-stripped LBPs incubated with bacterially expressed annexin A1 in the presence (With Ca^{2+}) or absence of Ca^{2+} (Without Ca^{2+}). (**E**) Quantification of annexin A1 fluorescence intensity on LBPs in the presence or absence of Ca^{2+}. The results are the means+s.d. for five independent experiments.

Cells with an annexin-A1-knockdown show impaired phagocytosis

Previous studies have shown that phagocytosis is impaired in macrophages isolated from annexin-A1-knockout mice (Hannon et al., 2003). In order to analyse in more detail the role of annexin A1 in phagocytosis, annexin A1 was downregulated using small interfering RNA (siRNA) in cultured RAW 264.7 macrophages.

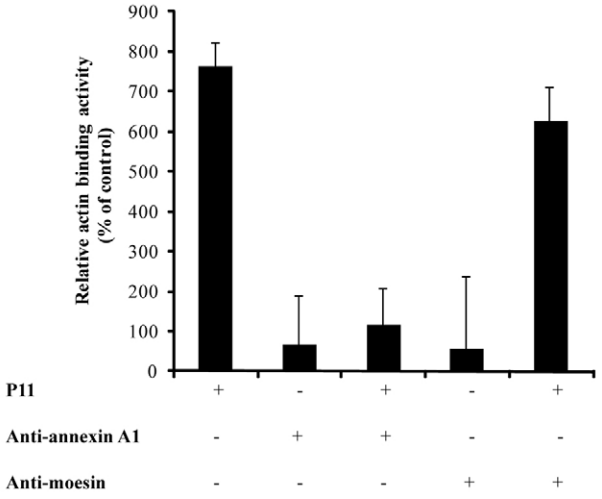

Fig. 5. Inhibition of actin-binding activity using specific antibodies. Relative F-actin–LBP binding activity of phosphocellulose-eluted proteins (P11) alone or with phosphocellulose-eluted proteins pre-incubated with anti-annexin A1 or anti-moesin antibodies. The results are the means+s.d. for three independent experiments.

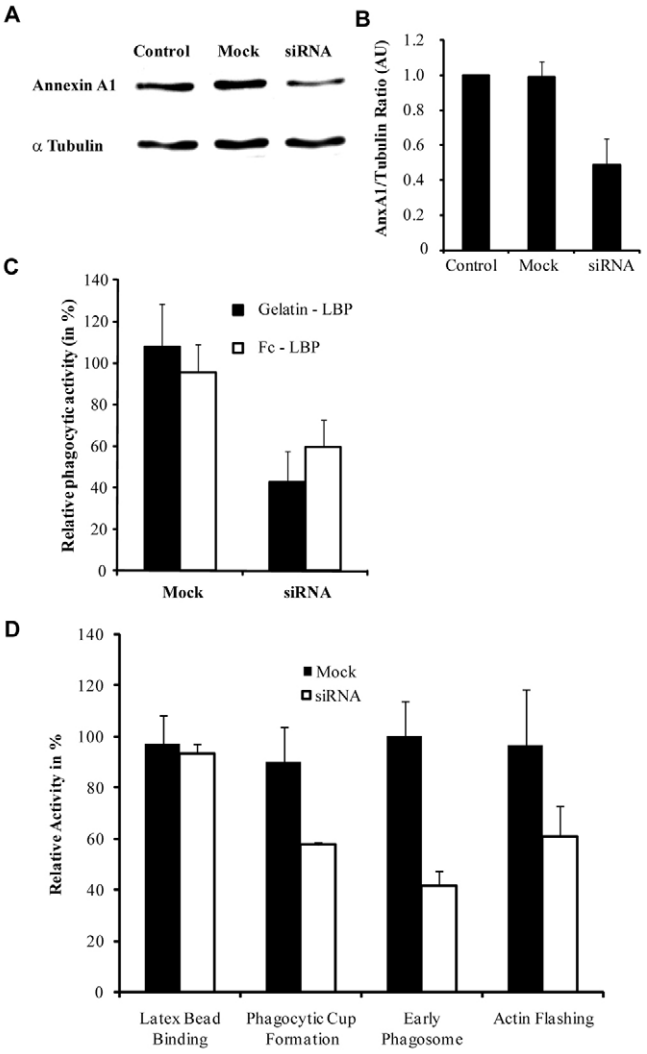

Fig. 6. Knockdown of annexin A1 impairs the phagocytosis of cells. (**A**) Immunoblotting of the cell lysates prepared from control cells (Control), mock-transfected cells (Mock) and cells transfected with siRNA targeting annexin A1 (siRNA) with anti-annexin A1 and anti-α-tubulin antibodies. (**B**) Quantification of annexin A1 expression data obtained by immunoblotting. The results are the means+s.d. for three independent experiments. (**C**) Relative phagocytic activity for gelatin- or Fc-coated latex beads (latex beads per cell) of transfected cells. The results are the means+s.d. for three independent experiments. (**D**) RAW 264.7 macrophages that were mock-transfected or transfected with siRNA targeting annexin A1 were incubated with 3-μm-diameter latex beads for different times (see the Materials and Methods section) to analyse binding of latex beads, phagocytic cup formation, early phagosome formation and actin flashing. Annexin-A1-knockdown did not affect binding of latex beads but significantly diminished other phagocytic activities.

The control and mock-transfected cells did not show any reduction in annexin A1 expression, whereas cells transfected with siRNA targeting annexin A1 showed a nearly 50% reduction in total annexin A1 levels (Fig. 6A,B). The phagocytic activity (number of latex beads per cell) of mock-transfected cells was comparable with that of control nontransfected cells (Fig. 6C). However, siRNA-transfected cells showed a nearly 50% reduction in their phagocytic activity (Fig. 6C).

Several studies have indicated that annexin-A1-knockout reduces the expression of some phagocytic receptors in mouse macrophages (Hannon et al., 2003; Yona et al., 2004). Therefore, we checked whether the reduction of phagocytic activity in annexin-A1-depleted cells was a primary consequence of the reduced annexin A1 levels or whether it was a secondary effect resulting from a potential decrease in cell surface receptors. First of all, we addressed this by using the well-defined FcγR-mediated phagocytic pathway, because it is known that the expression of FcγRs is not affected in annexin-A1-knockout macrophages (Yona et al., 2004). The experiments were performed using 1-μm-diameter latex beads coated with mouse IgG Fc fragments (Fc beads), which, as shown previously, internalise specifically through FcγRs (Ahmad et al., 2010; Hoffmann et al., 2010). Although the efficiency of the phagocytosis of the Fc beads was nearly 2.5 times higher than that of gelatin-coated beads (data not shown), RAW 264.7 macrophages transfected with siRNA targeting annexin A1 also had a significantly diminished phagocytic activity (~40%; Fig. 6C). These results indicate that annexin A1 is involved in the phagocytic uptake of beads and that the reduced phagocytosis in annexin-A1-depleted cells is not due to a possible effect on receptor expression.

Next, we investigated the role of annexin A1 in four major events of phagocytosis: ligand–receptor binding, phagocytic cup formation, early LBP formation and 'actin flashing' of late LBPs. RAW 264.7 macrophages transfected with siRNA targeting annexin A1 were incubated with 3-μm-diameter latex beads for different times and the binding and uptake of beads were analysed as described in the Materials and Methods section. Annexin-A1-knockdown did not affect the binding of latex beads to RAW 264.7 macrophages (Fig. 6D) but resulted in impaired phagocytic cup formation (Fig. 6D). The reduction in annexin A1 expression also resulted in a nearly 60% decrease in early phagosome formation (Fig. 6D). Furthermore, siRNA knockdown experiments revealed that the reduction in annexin A1 expression diminished the transient F-actin assembly on late LBPs, which is also referred to as the actin flashing phenomena (Liebl and Griffiths, 2009; Yam and

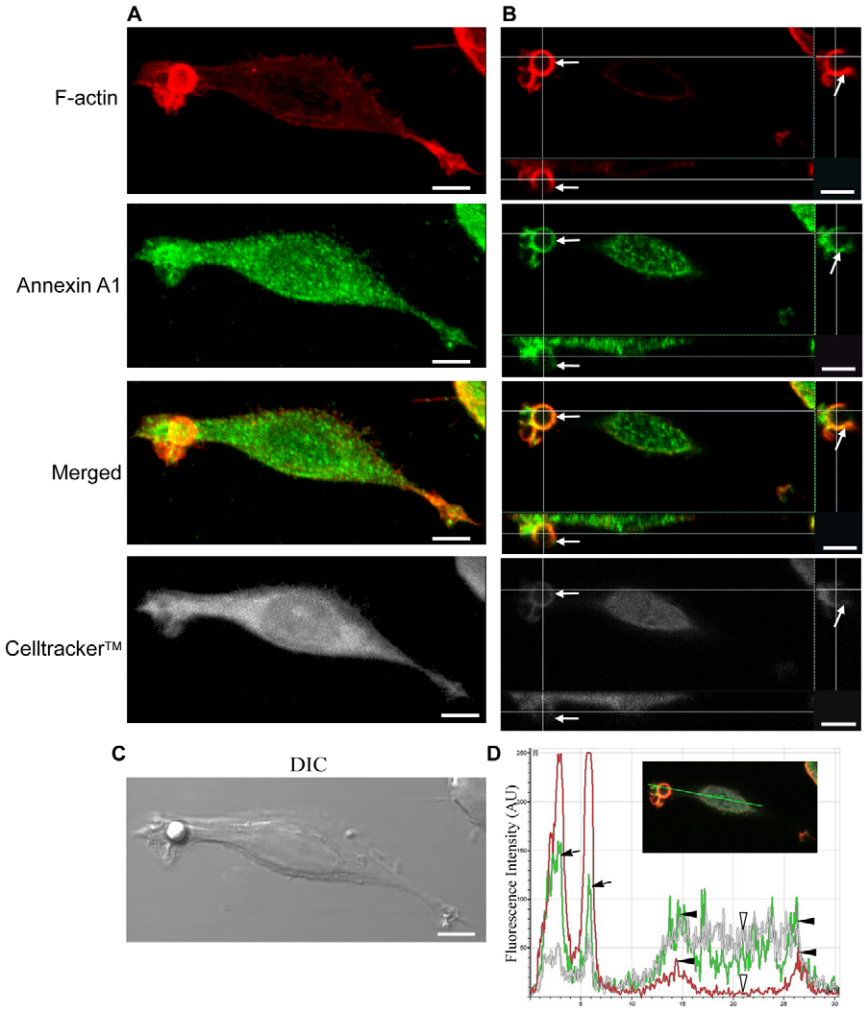

Fig. 7. Colocalisation of LBPs and annexin A1 during the early stages of phagocytosis. RAW 264.7 cells were incubated with latex beads in internalisation medium for 5 minutes, followed by fixing and staining. Cells were stained for annexin A1 (green), F-actin (red) and a cytosolic marker (CellTracker; in grey). (**A**) Maximum intensity projection of confocal images. (**B**) Three-dimensional cross-section images of phagocytic cup formation. Annexin A1 colocalised with F-actin on the extended filopodia of phagocytic cups (arrows). (**C**) A differential interference contrast microscopy image shows the position of the latex bead. (**D**) A section of a confocal image analysed for the fluorescence profile. The histogram shows the fluorescence intensities of F-actin (red line), annexin A1 (green line) and CellTracker (grey line). A higher annexin A1 concentration can be seen at the phagocytic cups (arrows) compared with in the cytosol (open arrowheads) and the cell membrane (filled arrowheads). Scale bars: 5 µm.

Theriot, 2004). Following a pulse–chase regimen of siRNA-mediated reduction of annexin A1 expression (1-hour pulse and 2-hour chase), there was a nearly 40% decrease in actin flashing events at the LBP (Fig. 6D).

Spatial and temporal colocalisation of annexin A1 and F-actin during phagocytosis

Given that annexin-A1-knockdown had a strong inhibitory effect on phagocytic events where F-actin dynamics play a key role, we studied the spatial and temporal dynamics of the association of annexin A1 with LBPs during the phagocytic events described above. Because actin polymerisation is a major and indispensable step of phagocytic cup formation (Castellano et al., 2001) and annexin A1 is an ABP possibly involved in linking F-actin to nascent phagosomes, we analysed colocalisation of annexin A1 and F-actin at phagocytic cups. Macrophages were triple stained with phodamine–phalloidin for F-actin, anti-(annexin A1) antibodies and with the cytosolic marker CellTracker. As expected, strong fluorescent staining of F-actin was observed at the sites of latex bead uptake at the cell periphery (Fig. 7A,C). By contrast, both annexin A1 and CellTracker appeared to be more or less evenly distributed in the cytosol (Fig. 7A). Therefore, a three-dimensional cross-section analysis of confocal images was performed (Fig. 7B). This analysis clearly showed that annexin A1 colocalised with F-actin on phagocytic cups (Fig. 7B, arrows). To obtain further insight, we analysed the fluorescence intensity of the individual dyes in the confocal images (Fig. 7D). Along with F-actin, the intensity of annexin A1 staining was found to be higher on phagocytic cups (Fig. 7D, arrows) compared with the staining intensity of other cell regions, such as the cytoplasm (Fig. 7D, open arrowheads) and plasma membrane (Fig. 7D, filled arrowheads). Subsequently, we analysed the fluorescence intensity ratio (fluorescent intensity at phagocytic cup over that of the cytosol) for annexin A1 and CellTracker. The intensity ratio of annexin A1 at phagocytic cups was two times higher than in cytosol (s.d.=0.42; n=50), whereas that of CellTracker was only 0.8 times (s.d.=0.23; n=50). These data clearly suggest that, in a manner similar to F-actin, annexin A1 also accumulated on the phagocytic cups.

Naive LBPs acquire a dense F-actin network on the membrane, which gradually decreases as they become more mature (Castellano et al., 2001). Indeed, after uptake we observed F-actin accumulation (Fig. 8A, arrowhead) and then F-actin dissociation on LBPs (Fig. 8A, arrows). Notably, both events were accompanied by an increase and then decrease of LBP-associated annexin A1 (Fig. 8A). Furthermore, as described above, at late stages of phagocytosis some LBPs show actin flashing (Liebl and Griffiths, 2009; Yam and Theriot, 2004). During the actin flashing, a prominent annexin A1 accumulation was also detected near late LBPs (Fig. 8B).

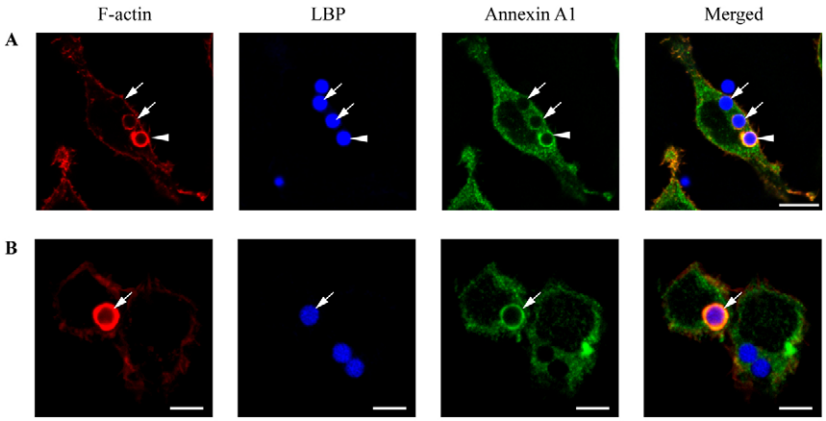

Fig. 8. Annexin A1 colocalises with F-actin at the early and late stages of phagocytosis. RAW 264.7 cells were incubated with latex beads in internalisation medium for (**A**) 30 minutes or (**B**) a 60 minute pulse followed by an 120 minute chase. Annexin A1 (green) colocalised with F-actin (red) on an early LBP (blue; A, arrowhead) and on LBP displaying transient actin flashing (blue; B, arrow). The accumulation of annexin A1 on LBPs eventually decreased as LBP matured (A, arrows). Scale bars: 5 µm.

Taken together, our results indicate that annexin A1 is spatially and temporally colocalised with F-actin on phagosome membranes during early and late stages of phagocytosis.

Discussion

Once recognised by the cell surface receptors, internalisation and subsequent intracellular transport of phagocytic particles require a highly regulated and dynamic interaction of the phagosome membrane with cytoskeletal elements. Particle engulfment requires extensive remodelling of the membrane and actin cytoskeleton (May and Machesky, 2001). The newly formed phagosome possesses a dense coat of actin and ABPs, which subsequently decreases with time and is eventually lost (Castellano et al., 2001; Greenberg et al., 1990; May and Machesky, 2001). Although the signalling pathways leading to F-actin polymerisation during phagocytosis have been extensively studied (Allen and Aderem, 1996; Castellano et al., 2001; Diakonova et al., 2002), the mechanics of actin dynamics during phagosome formation, and particularly the role of ABPs, remain poorly understood. Over the past few years our group has been focusing on the identification of the ABPs involved in the phagosome–F-actin interaction. In a previous study, we had shown that the unconventional myosin Va is important for proper phagosome inward transport at the post-internalisation stages of phagocytosis (Al Haddad et al., 2001). Here, we identified annexin A1 as a new ABP responsible for mediating phagosome interactions with the actin cytoskeleton.

In order to search for new players in phagosome biogenesis, we used the well-established latex bead system and developed an in vitro light-microscopy assay to study phagosome–F-actin interactions (Al Haddad et al., 2001). LBPs loaded with non-degradable fluorescent latex beads provide several unique advantages when studying molecular interactions between a defined membranous organelle and the cytoskeleton (Al Haddad et al., 2001; Desjardins and Griffiths, 2003). One of the most important advantages is that LBPs can be isolated (>95% purity) in a single-step procedure by flotation on a discontinuous sucrose gradient (Desjardins et al., 1994b; Desjardins et al., 1994a; Desjardins and Griffiths, 2003; Stuart et al., 2007). Our F-actin–LBP binding assay has also several important advantages. It is simple, easy to manipulate, robust and requires minimal amounts of actin, LBPs and test probes (Al Haddad et al., 2001).

By using this assay, we were able to show that the macrophage cytosol stimulated the F-actin–LBP interaction in vitro at low protein concentrations (~0.5 mg/ml). However, isolation of biochemically pure individual factors was not possible, owing to the limited availability of macrophage cytosol as a starting material. Therefore, as a first step, we decided to fractionate macrophage cytosol and isolate a fraction enriched in F-actin–LBP binding activity, and we then identified potential candidates by LC-MS/MS. This resulted in the identification of 11 ABPs and, among these, annexin A1 was determined to be the most likely to be involved in the F-actin–LBP interaction.

Annexin A1 binds and bundles F-actin in a Ca^{2+}-dependent manner, with a high Ca^{2+} concentration essential to establish this interaction in vitro (Glenney, Jr et al., 1987; Schlaepfer and Haigler, 1987). Bacterially expressed recombinant annexin A1 requires 1 mM Ca^{2+} for actin binding in an in vitro assay (Fig. 3), which is consistent with a previous finding (Glenney, Jr et al., 1987). However, the regulatory role of Ca^{2+} in mediating the interaction of annexin A1 and F-actin inside the cell is not clear. Given that it has been reported previously that the presence of phospholipids or accessory molecules markedly reduces the affinity of annexins towards Ca^{2+} (Powell and Glenney, 1987; Schlaepfer and Haigler, 1987), we suggest the existence of different mechanisms for F-actin and annexin A1 interaction in vivo. The stimulation of the F-actin–LBP interaction by gel-filtration- and phosphocellulose-eluted protein fractions, in the absence of Ca^{2+}, supports this hypothesis (Fig. 1A and Fig. 2A). However, addition of Ca^{2+} to these fractions further increased their F-actin–LBP binding activity (data not shown). Therefore, the role of Ca^{2+} in regulating the interaction between F-actin, annexin A1 and LBPs during phagocytosis requires further investigation.

Most annexins are abundant intracellular proteins (Raynal and Pollard, 1994), and in polymorphonuclear leukocytes (PMNs) annexin A1 comprises ~2–4% of total cellular protein, which is almost half of the amount of actin (Lim and Pervaiz, 2007; Perretti and Flower, 1996; Raynal and Pollard, 1994). In PMNs and monocytes, ~17% and ~33%, respectively, of total annexin A1 is associated with the plasma membrane (Perretti and Flower, 1996). From our immunofluorescence analysis, we could also clearly see that there was a high level of annexin A1 both in the cytoplasm and in ruffles of the plasma membranes (Fig. 8). The fact that annexin A1 was colocalised with F-actin at the plasma membrane, filopodia and ruffles, indicates that annexin A1 could play a role during phagocytic cup formation and particle uptake.

In support of the above hypothesis, the present siRNA data and previous knockout studies demonstrate the importance of annexin A1 for phagocytic activity (Hannon et al., 2003). RAW 264.7 macrophages transfected with siRNA targeting annexin A1 showed at least a 50% reduction in annexin A1 expression and a similar

reduction in the phagocytic activity (Fig. 6). In addition, it has been shown that peritoneal macrophages from annexin-A1-knockout mice have impaired phagocytosis (Yona et al., 2004; Yona et al., 2006) and a lower level of internalisation of zymosan, correlating with a higher number of zymosan particles that remained bound to the cell surface (Yona et al., 2004). Previous studies (Hannon et al., 2003; Yona et al., 2004) have suggested that annexin-A1-knockdown reduced the expression of several receptors and cell membrane markers. However, in the present experiments, annexin-A1-knockdown did not affect the capacity of cells to bind latex beads to their cell surface. Furthermore, the phagocytosis of Fc-coated latex beads was significantly impaired following knockdown of annexin A1 (Fig. 6D), although the expression of the participating FcγRs is not affected. Collectively, our data suggest that the observed reduction in macrophage phagocytic activity was due to the reduction in annexin A1 levels and not due to a secondary decrease in cell surface receptors. Interestingly, neither knockout nor knockdown of annexin A1 completely abolished the phagocytic activity of macrophages, indicating the existence of an alternative, but less effective, mechanism for phagocytosis. Moreover, given that the core domain (containing the actin-binding site) from different annexins have a high similarity and that removal of the N-terminal domain does not alter the association of annexin A1 with phagosomes in J774A.1 cells (Kusumawati et al., 2001), it is tempting to speculate that one of the annexin protein family members, probably annexin A2, takes over its role in phagocytosis.

The molecular mechanism by which membrane-bound annexin A1 modulates the actin cytoskeleton reorganisation has yet to be discovered. However, some reports have already established an association of annexin A1 with F-actin (Diakonova et al., 1997; Glenney, Jr et al., 1987; Kusumawati et al., 2001; Schlaepfer and Haigler, 1987). On the basis of these previous studies and the present findings, we propose that phospholipids could play an important role in the spatial–temporal recruitment of annexin A1 to cell membranes from cytoplasm. We suggest that a transient increase in the phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] and phosphatidic acid (PtdOH) concentration at the phagocytic sites (Coppolino et al., 2002; Scott et al., 2005) stimulates annexin A1 recruitment and its accumulation on membranes during phagocytic cup formation (Fig. 7B). The recruited annexin A1 could link the growing F-actin to the plasma membrane in order to generate enough support and strength for membrane protrusions and phagocytic cup formation. This hypothesis is supported by fact that annexin-A1-knockdown in macrophages impaired their ability to form phagocytic cups (Fig. 6D).

Soon after the particle internalisation, the concentrations of PtdIns(4,5)P_2 and PtdOH on the phagosome membrane decreases to the basal cellular level (Yeung and Grinstein, 2007). This might correlate with a significant decrease of annexin A1 on the phagosome surface (Fig. 7B, arrows) and a following F-actin dissociation, which is believed to be an important process for the maturation of newly formed phagosomes (Liebl and Griffiths, 2009; May and Machesky, 2001). By contrast, annexin-A1-knockdown does not affect the recruitment of the late phagocytic marker LAMP-2 to LBPs (data not shown). Taken together, we suggest that annexin A1 is not directly involved in F-actin assembly–disassembly but provides structural support by linking membrane and F-actin during the particle internalisation.

Even at the late stages of phagocytosis, annexin A1 was found to be associated with LBPs in vitro (Fig. 4A), which is consistent with previous findings (Diakonova et al., 1997). Recently, it has been reported that a transient actin flashing occurs on phagosomes at the later stages of phagocytosis (Liebl and Griffiths, 2009). We found that annexin A1 was also present on the LBPs displaying actin flashing (Fig. 8B). The accumulation of annexin A1 on LBPs displaying flashing strongly correlated with the accumulation of F-actin, and a functional involvement of annexin A1 in actin flashing was confirmed by annexin-A1-knockdown, which dramatically reduced actin flashing on LBPs. The reduction in actin flashing was proportional to the reduction in annexin A1 expression, with a 50% reduction of annexin A1 resulting in a nearly 40% reduction in actin flashing events (Fig. 6D). These data once again indicate that annexin A1 is a functional linker between LBPs and F-actin during both the early and late stages of phagocytosis. However, further investigation is needed to understand in more detail the mechanism underlying the role of annexin A1 in linking actin filaments to LBPs. One cannot exclude the involvement of additional unknown factor(s) in this process.

Interestingly, our fractionation studies revealed that the cytosol most probably contains not only factors stimulating F-actin–LBP linkage, such as annexin A1 and myosin Va (Al-Haddad et al., 2001), but also unknown inhibitory factor(s). Owing to the presence of these inhibitory factors, annexin A1 present in the phosphocellulose-unbound protein fraction (Fig. 2B) could not stimulate F-actin–LBP binding (Fig. 2A). The presence of these inhibitory factor(s) was confirmed by F-actin–LBP binding experiments carried out with recombinant annexin A1 in the absence or presence of aliquots of the phosphocellulose-unbound protein fraction. These revealed a decrease in the annexin A1 stimulatory activity, by nearly 60%, in the presence of the unbound protein fraction (data not shown). The identification of this inhibitory factor(s), which could play an important role in the regulation of the annexin-A1–LBP–F-actin interaction, will be a specific aim of future studies.

Materials and Methods

Cells, latex beads and antibodies

J774.A1 mouse macrophage cells were obtained from the German Resource Centre of Biological Material (DSMZ) Braunschweig, Germany, and were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Biochrom, Germany) at 37°C and under 5% CO_2. RAW 264.7 mouse macrophages were obtained from the American Type Culture Collection (Manassas, VA) and were maintained as described previously (Al Haddad et al., 2001). Nile-Blue-conjugated (emission at 350 and 440 nm) 1-μm-diameter fluorescent carboxylated latex beads (Invitrogen) and 3-μm-diameter nonfluorescent carboxylated beads (Polysciences) were conjugated to fish-skin gelatin (Sigma–Aldrich) and Fc fragments of mouse IgG (Thermo Scientific) as per the manufacturer's recommendations. Unless otherwise stated, gelatin-coated latex beads were used in the present study. The anti-(annexin A1) antibodies were produced in rabbit (Seemann et al., 1996), the anti-annexin A2 monoclonal antibodies were as described previously (Thiel et al., 1991), the rabbit polyclonal anti-moesin antibody was from Cell Signaling, the mouse monoclonal anti-α-tubulin was from Sigma–Aldrich and all secondary antibodies were purchased from Invitrogen.

Preparation of cytosol

Cytosol was prepared from J774A.1 cells cultured on 24.5-cm-diameter cell culture plates until they reached 80–90% confluency. Cells were harvested from 10 to 15 cell culture plates by trypsinisation. Cells were collected by centrifugation at 2500 ***g*** for 5 minutes and washed three times with ice-cold PBS. The cell pellet was re-suspended in HBKS [25 mM HEPES-KOH, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, 2 mM dithiothreitol (DTT) and 10% sucrose] and re-pelleted by centrifugation for 5 minutes at 2500 ***g*** at 4°C. Cells were re-suspended in 0.4 volumes of HBKS plus protease inhibitors and homogenised by passage through a 24-gauge syringe needle until 90–95% of the cells were lysed. The homogenate was then centrifuged for 30 minutes at 40,000 ***g***. The supernatant was removed and centrifuged for 1 hour at 150,000 ***g*** at 4°C, to generate cytosol. Cytosol preparations from J774A.1 cells typically contained 30–35 mg of protein/ml, as

measured by the method of Bradford (Bradford, 1976). Cytosol preparations were flash-frozen in liquid nitrogen and stored at –80°C.

Electrophoresis, immunoblotting and silver staining

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) on 12% polyacrylamide gels. Proteins were stained with a silver staining kit (Thermo Scientific). Molecular mass markers were from Bio-Rad. For immunoblotting, proteins were transferred onto nitrocellulose membranes (Whatman) at a constant 30 V overnight using a tank blotting device (Bio-Rad, Germany). The membrane was blocked in 5% non-fat dried milk powder and labelled according to the method described previously (Towbin et al., 1979), with the use of horseradish-peroxidase-labelled goat anti-(rabbit Ig) secondary antibodies, and was then visualised with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Chemiluminescent signals were developed and visualised on Kodak MR Films (Sigma–Aldrich).

Cytosol fractionation

The cytosol was fractionated by size-exclusion chromatography with the use of a Bio-Silect SEC 400-5 column on a Bio-Rad Biologic workstation. Usually, 7–10 mg of cytosolic protein (~250 µl) was loaded onto columns pre-equilibrated with HB (25 mM HEPES-KOH, pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA and 2 mM DTT) and run at flow rate of 0.5 ml/minute. Fractions (250 µl) were collected and protein-containing fractions were assayed to determine the F-actin–LBP binding activity. Fractions showing the highest actin-binding activity were pooled together. For cation chromatography, 50–100 µl of the pooled fractions was incubated with 10 µl of phosphocellulose resin (P11), which had been pre-equilibrated with HB, for 20 minutes at 4°C. After two washes with HB, bound proteins were eluted with 50–100 µl of 1 M NaCl prepared in HB. The eluted fraction was dialysed against the HB before use in the actin-binding assay.

Isolation of phagosomes

Cells were incubated in internalisation medium (serum-free DMEM containing 10 mM HEPES, pH 7.4), with 0.02% latex beads coupled to fish-skin gelatin, on a shaker for 1 hour (pulse) followed by brief washing in warm PBS and further incubation in complete medium for 1 hour (chase). Phagosomes containing latex beads were isolated by sucrose-gradient flotation and salt-stripped as described previously (Al Haddad et al., 2001; Desjardins et al., 1994a). Unless stated otherwise, salt-stripped phagosomes were used in all experiments. The number of phagosomes in any preparation was determined by measuring the attenuance of the preparations at 600 nm with the use of the extinction coefficient for 1% latex beads (0.01 g/ml; ε_{600}=1000).

Actin-binding assay

The actin-binding assay was performed as described previously (Al Haddad et al., 2001). Briefly, microscope chambers were built from a glass microscope slide and an 11-mm-diameter circular glass coverslip forming a 2–3-µl chamber. F-actin, stabilised and labelled with rhodamine–phalloidin, was perfused into the chamber and incubated for 5 minutes. Nonspecific binding was blocked by perfusion with 3 mg of casein/ml (Sigma–Aldrich) prepared in HB. Excess F-actin and casein were washed away by perfusion with three chamber volumes of HBS (HB containing 10% sucrose), containing 0.3 mg of casein/ml. The phagosome-binding reaction mixture (8–10 µl), containing LBPs (working concentration ~0.001% wt/vol), 0.3 mg of casein/ml and different binding factors to be tested, were perfused into the chamber and incubated for 20 minutes. All incubations were performed in a moist chamber at room temperature. Unbound LBPs were washed away by perfusion with three volumes of HBS containing 0.3 mg of casein/ml. Binding was analysed by fluorescence microscopy with the use of a Nikon Diaphot 300 microscope (Nikon). The number of bound phagosomes per field was counted (field surface area of ~22,000 μm^2) and values from at least 20 fields were averaged. Annexin A1 was purified using the pKK Anx A1 expression vector as described previously (Seemann et al., 1996). To assess the in vitro actin-binding activity of purified annexin A1, annexin A2 (a kind gift from Jean Gruenberg and Etienne Morel (Morel and Gruenberg, 2009) or the annexin-A2–S100A10 complex (Gerke and Weber, 1984), 100 ng of protein was incubated in the presence or absence of 1 mM Ca^{2+}. For the experiments with antibodies, the phosphocellulose fraction was incubated with anti-(annexin A1) and anti-moesin rabbit polyclonal antibody for 20 minutes before use in the actin-binding assay. The errors reported are the s.d. of at least five independent repeats. The data presented are the means for five independent experiments.

Liquid chromatography tandem mass spectrometry

Phosphocellulose-eluted proteins were precipitated using ammonium sulfate at final concentration 80%. The precipitated proteins were collected by centrifugation. The pellet was dissolved and digested with trypsin (Promega) for 24 hours at room temperature, followed by another 24-hour digestion after addition of fresh trypsin. The LC-MS/MS procedure was performed by the W. M. Keck Biomedical Mass Spectrometry Laboratory (University of Virginia, Charlottesville, VA, USA). Approximately 25% of the digest was introduced into the mass spectrometer for analysis using two run conditions. The data obtained were analysed by database searching using the Sequest search algorithm against the mouse IPI accessions and Swiss-Prot database. Identified proteins from each sample were analysed using Scaffold software (Proteome Software) applying a minimum protein identification probability of 85%.

Immunofluorescence microscopy

For immunofluorescence microscopy, LBPs incubated with different reagents in HB were layered on top of 1.5 ml of HB buffer containing 5% sucrose and centrifuged onto coverslips at 9000 ***g*** for 10 minutes at 4°C. The LBPs on coverslips were then fixed in 4% paraformaldehyde (PFA) for 20 minutes, followed by quenching with 50 mM NH_4Cl for 10 minutes and then washing with PBS. Fixed LBPs were blocked with 1% gelatin ‘gold’ (Sigma–Aldrich) and labelled with rabbit polyclonal anti-(annexin A1) antibody (Seemann et al., 1996) at a 1:150 dilution in 1% gelatin gold followed by goat anti-(rabbit Ig) conjugated to Alexa Fluor 488 (Invitrogen, Germany) at 1:300 in 1% gelatin gold. The intensity of annexin A1 on LBPs, in the presence or absence of Ca^{2+}, was quantified from 25 different LBPs. J774A.1 and RAW 264.7 macrophages were labelled in a manner similar to LBPs, except cells were permeabilised with 0.1% saponin prepared in PBS and then washed twice with PBS. F-actin was labelled with rhodamine-conjugated phalloidin (Sigma–Aldrich) at a 1:500 dilution in 1% gelatin gold for 30 minutes. All preparations were analysed using confocal laser-scanning microscopy (TCS SP2, Leica Microsystems, Germany). To study the role of annexin A1 in phagocytosis, RAW 264.7 macrophages were incubated with 3-µm-diameter latex beads for pulse–chases in minutes of: 5–0, 15–0, 30–0, 60–0, 60–60, 60–120 and 60–240, followed by fixation and staining as described above. Between the pulse and chase, cells were washed three times with warm PBS to remove any unbound latex beads. To evaluate the accumulation of annexin A1 on phagocytic cups and early LBPs, cells were stained with the cytosolic marker CellTracker Violet BMQC (Invitrogen, Germany) according to the manufacturer’s instructions. Stained cells were incubated with 3-µm-diameter latex beads for 5 minutes at 37°C followed by immunofluorescence staining as described above. After confocal scanning, images were evaluated for fluorescence profiles using the Leica image analysis software (Leica Microsystems, Germany). The software provides histograms for each fluorescence dye used in a given confocal plane. To evaluate the accumulation and to retrieve quantitative data, we compared the estimated fluorescence intensity ratio at the phagocytic cup with that in cytosol for annexin A1 and CellTracker. To obtain statistically significant data, the ratio was evaluated for 15 phagocytic cups and in at least three confocal planes for each phagocytic cup (a total of 50 individual planes).

Knockdown of annexin A1 in cells

A total of 10,000–15,000 RAW 264.7 cells were seeded into 24-well cell culture plates. Next day, RAW 264.7 cells were transfected with siRNA targeting annexin A1 (SC-29682; Santa Cruz Biotechnology) and control siRNA (SC-37007) using siRNA transfection reagent (SC-29528) according to the manufacturer’s instructions. At 72 hours after transfection, cells were incubated with 3-µm- or 1-µm-diameter latex beads for 30 minutes in internalisation medium. After 30 minutes, cells were washed twice with ice-cold PBS and stained as described above. For immunoblotting, cell lysates were prepared using lysis buffer (SDS protein sample loading buffer without Bromophenol Blue). After immunoblotting, the intensity of protein bands was quantified using ImageJ software (NIH, USA). To exclude any loading error, the annexin A1:α-tubulin ratio was used to compare immunoblotting data.

In order to calculate the phagocytic activity, cells were incubated with 1-µm-diameter latex beads for 30 minutes and the total number of latex beads per cell was counted for at least 50 cells. The cellular location of the beads was analysed by subjecting the confocal images to three-dimensional cross-sectioning using the Leica image analysis software or the Imaris confocal analysis software (Bitplane Scientific Software, Switzerland). To evaluate the binding of the latex beads to macrophages and phagocytic cup formation activity, RAW 264.7 macrophages were incubated with 3-µm-diameter latex beads for 5 minutes, followed by extensive washing with ice-cold PBS to remove any unbound beads and to stop phagocytic activity. Subsequently, cells were fixed and stained as described above. The formation of filopodia or phagocytic cups was evaluated for each bead bound to the macrophages (minimum 50 cells) by three-dimensional cross-sectioning of the F-actin-stained cell. To study the formation of early phagosomes, macrophages were incubated with 3-µm-diameter latex beads for 15 minutes followed by washing and staining for F-actin as described above. For purpose of evaluation, latex beads, which are completely internalised and still harbour dense actin networks, were considered as early phagosomes (e.g. Fig. 8A, arrowhead). A minimum of 50 cells were evaluated for each transfection. To obtain statistically significant data for actin flashing events, the number of internalised and F-actin-positive LBPs per cell was counted from 500 cells. The data presented are means of three independent experiments and were subjected to Student’s *t*-tests in order to confirm that the stimulatory and inhibitory effects were statistically significant (P<0.01).

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