Regulation of podosome dynamics by WASp phosphorylation: implication in matrix degradation and chemotaxis in macrophages

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Summary
Podosomes, adhesion structures capable of matrix degradation, have been linked with the ability of cells to perform chemotaxis and invade tissues. Wiskott-Aldrich Syndrome protein (WASp), an effector of the RhoGTPase Cdc42 and a Src family kinase substrate, regulates macrophage podosome formation. In this study, we demonstrate that WASp is active in podosomes by using TIRF-FRET microscopy. Pharmacological and RNA interference approaches suggested that continuous WASp activity is required for podosome formation and function. Rescue experiments using point mutations demonstrate an absolute requirement for Cdc42 binding to WASp in podosome formation. Although tyrosine phosphorylation was not absolutely required for podosome formation, phosphorylation did not regulate the rate of podosome nucleation and actin filament stability. Importantly, WASp tyrosine phosphorylation does not alter WASp activation, instead phosphorylation appears to be important for the restriction of WASp activity to podosomes. In addition, the matrix-degrading ability of cells requires WASp phosphorylation. Chemotactic responses to CSF-1 were also attenuated in the absence of endogenous WASp, which could not be rescued with either tyrosine mutation. These results suggest a more complex role for tyrosine phosphorylation than simply in the regulation of WASp activity, and suggest a link between podosome dynamics and macrophage migration.

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Introduction
Podosomes are adhesion structures typical of monocyte-derived cells such as macrophages, dendritic cells (DCs) and osteoclasts (Linder and Aepfelbacher, 2003), although they have also been described in lymphocytes (Carman et al., 2007; Redondo-Muñoz et al., 2006), endothelial cells (Moreau et al., 2003) and A7r5 vascular smooth muscle cells (Gimona et al., 2003). Structurally, they consist of a filamentous (F)-actin-rich core that also contains actin-regulatory proteins including Wiskott-Aldrich syndrome protein (WASp), cortactin and Arp2/3, and a surrounding ring containing cytoskeletal components typical of focal contacts, such as talin andvinculin (Linder and Aepfelbacher, 2003). The intervening space between podosomes is suggested to contain a ‘cloud’ of loose F-actin bundles that might link individual podosomes to each other, as well as with the plasma membrane, the formation of which is regulated by Src (Destain et al., 2008). β1, β2 and β3 integrins are variably associated with the ring or the core (Linder and Aepfelbacher, 2003), and mediate adhesion to the extracellular matrix (ECM).

Secretion of matrix metalloproteases (MMPs) renders podosomes capable of degrading and remodelling the ECM (Linder, 2007). Indeed, osteoclasts are professional cells involved in bone resorption, and their podosomes are arranged in unique clusters that form a peripheral belt – the sealing zone – when in contact with mineralised ECM. In macrophages and other cell types, this organisation is absent, yet podosomes are generally observed in clusters and are typically associated with the periphery of cells cultured in 2D.

WASp is a haematopoietic cell-specific protein involved in de novo actin polymerisation via the Arp2/3 complex (Takenawa and Suetsugu, 2007) and is required for podosome formation (Linder et al., 1999). In resting cells, WASp and N-WASP [referred to collectively as (N)WASp] are mostly in a closed autoinhibitory conformation owing to intramolecular interactions of the C-terminal verprolin-homology, coflin-homology, acidic (VCA) domain with the basic and G-protein-binding (GBD) domains (Kim et al., 2000), preventing the interaction of the VCA region with actin and the Arp2/3 complex. For this reason, the interaction of the WASp GBD with Cdc42 is considered to be an important step in the unfolding and activation. PtdIns(4,5)P₂ acts synergistically in the activation of (N)WASp by Cdc42 (Higgs and Pollard, 2000; Rohatgi et al., 2000), even though a recent study reported an inhibitory effect of PtdIns(4,5)P₂ on WASp, but not N-WASP, activity (Tomassević et al., 2007). Importantly, the GBD domain contains the major tyrosine phosphorylation site on WASp (Y291), because mutation of this residue abolishes pervanadate-induced tyrosine phosphorylation of WASp (Cammer et al., 2009; Cory et al., 2002).
The role of phosphorylation of this residue, as well as the interplay between GTPase signals, has been studied in detail in vitro (Suetsugu et al., 2002; Torres and Rosen, 2003; Torres and Rosen, 2006). Phosphorylation of Y291 by Src-family kinases (SFKs) or a Y291E phosphomimetic mutant increased the rate of actin polymerisation in vitro, whereas mutation of this residue (Y291F) exhibited similar kinetics to the nonphosphorylated protein. Despite this, the exact role of (N)WASP tyrosine phosphorylation in vivo remains unclear. In addition to SFKs, Abl, FAK, ACK1, Btk and Pyk2 have been found to interact with and/or phosphorylate (N)WASP in vitro, or when overexpressed in cells (Badour et al., 2004; Burton et al., 2005; Cory et al., 2002; Gross et al., 1999; Guinamard et al., 1998; Wu et al., 2004; Yokoyama et al., 2005). Endogenous WASP phosphorylation has been detected following TCR, collagen receptor glycoprotein-VI, integrin or CD16 engagement with ligand or antibodies (Badour et al., 2004; Chellaiah et al., 2007; Gismondi et al., 2004; Lutskiy et al., 2007; Oda et al., 1998; Soriani et al., 2006). However, the significance of this event remains uncertain. Microinjection of the Y291E WASP mutant resulted in increased filopodia formation in macrophages (Cory et al., 2002). Alternatively, (N)WASP phosphorylation might signal the proteasome-mediated degradation of the protein (Suetsugu et al., 2002), regulate its intracellular localisation (Wu et al., 2004) or the assembly of macromolecular complexes (Chellaiah et al., 2007).

In this study, the role of WASp and the effect of the various mutations that interfere with Cdc42 binding and Y291 phosphorylation in the formation and regulation of podosomes, as well as in the chemotactic response, in macrophages was addressed. The data suggest that Cdc42 binding and activation of WASp are required for podosome formation and chemotaxis. Tyrosine phosphorylation, however, is required for efficient targeting of WASp activity in podosomes, and regulates podosome formation on a fibronectin substrate and podosome actin dynamics. However, interfering with the phosphorylation site adversely affects chemotaxis, suggesting a more complex interplay between podosomes, WASP phosphorylation and chemotaxis.

**Results**

**WASP activity is required for podosome formation and function**

For this study, the murine monocyte and macrophage RAW/LR5 cell line was used (Cox et al., 1997). A significant number of these cells spontaneously displayed cytoskeletal structures in their ventral surface that exhibited a typical F-actin-rich core surrounded by a vinculin-positive ring; they were homogeneous in size with a diameter of approximately 0.5 μm and were evenly distributed within clusters. Their characteristics were similar to podosomes of human primary monocyte-derived macrophages (Fig. 1A) and therefore meet all the criteria to be defined as genuine podosomes.

The localisation of WASP in macrophage podosomes has been previously demonstrated by immunofluorescence microscopy (Linder et al., 1999). Nevertheless, its activity status inside podosomes has not been determined. Using a WASp FRET-based biosensor (Cammer et al., 2009), the activity of WASP in podosomes was examined. Cells transfected with the WASp biosensor were examined using total internal reflection (TIRF) microscopy, allowing for the detection of signals deriving from the ventral adhesive part of the cell only. As shown in Fig. 1B, WASP was unfolded and therefore, active only in podosomes, not in other ventral areas of the cell, where it also localised. This indicates the highly localised nature of WASP activity in podosomes, but not other adhesion structures of macrophages.

To assess the importance of WASP activity in podosome formation, cells were treated with wiskostatin, a small molecule inhibitor of (N)WASP that stabilises the autoinhibited conformation (Peterson et al., 2004). Treatment of RAW/LR5 cells with this inhibitor at 5 μM, which was shown to inhibit activation of the WASP biosensor (Cammer et al., 2009), revealed a reduction in the number of cells displaying podosomes as a function of time (Fig. 1C). Taken together, these data indicate that continuous WASP activity is required for the steady state of these structures.

The role of WASP in the regulation of podosomes was further examined using a vector-based small hairpin (sh)RNA approach that resulted in a prolonged and considerable reduction of WASP expression (>90% of endogenous protein levels) (Fig. 2A). The chemotactic ability of cells was then tested using a Transwell assay in response to the macrophage growth factor CSF-1. WASP silencing in RAW/LR5 cells dramatically reduced migration toward the chemoattractant (Fig. 2B), similarly to reports of monocyte-derived macrophages isolated from WAS patients (Zicha et al., 1998). Nevertheless, WASP reduction did not affect dorsal ruffle formation in response to global CSF-1 stimulation (data not shown), a process regulated by WAVE2 (Abou Kheir et al., 2005). WASP silencing affected cell morphology, resulting in rounded cells and...
a significant diminution of the number of cells with podosomes (Fig. 2C). Silencing of endogenous WASp by shRNA in macrophages can therefore recapitulate the hallmarks of WAS macrophages.

To confirm that WASp was necessary for podosome formation in RAW/LR5 cells, human Myc-tagged WASp was stably introduced in cells depleted of endogenous WASp. After selection, several clones expressing variable levels of Myc-WASp were obtained, ranging from undetectable (A5) to significant expression (G11) (Fig. 2D). Progressive recovery of normal morphology in terms of cell elongation (not shown) and the presence of podosomes was observed as a function of the level of Myc-WASp expressed by the cells (Fig. 2E). Full rescue was achieved in the G11 clone, used in all subsequent experiments, that appeared virtually identical to control cells (henceforth referred to as wtWASp clone).

Cdc42 binding, but not Y291 phosphorylation, is required for podosome formation

The stable WASp-silencing system allowed us to question what are the key signals to WASp that regulate its function in podosomes. Cdc42 has been recognised as the primary WASp-activating signal, even though both constitutively active and dominant-negative Cdc42 have been suggested to disassemble podosomes in macrophages and DCs (Burns et al., 2001; Linder et al., 1999). For this reason, WASp bearing the point mutation H246D, which abolishes Cdc42 binding to WASp (Kato et al., 1999), was introduced to the WASp-silenced cells (Fig. 3A). The H246D mutant did not rescue the podosome defect induced by the WASp shRNA (Fig. 3B). Consistently, silencing Cdc42 also abolished podosome formation and this effect could be rescued by introduction of human CDC42 cDNA (Fig. 3C,D). Therefore Cdc42 binding appears to be a major regulatory signal in podosome formation by WASp.

As discussed, Y291 is the major phosphorylation site on WASp and affects actin polymerisation rates in vitro. For that reason, Myc-tagged WASp mutants bearing point mutations to Y291 to either mimic (Y291E) or abolish (Y291F) phosphorylation were introduced to the WASp-silenced cells. Clones that displayed comparable levels of expression to the wtWASp clone were selected (Fig. 4A). As with the wt WASp, the cells that re-expressed the Y291F and Y291E point mutants could restore most of the morphological defects found in shWASp cells. Therefore, both the number of cells displaying podosomes and their elongated morphology were restored (Fig. 4B,C). Despite the apparent rescue resulting from the expression of WASp Y291E/F mutants, closer investigation revealed the presence of more podosomes for the Y291E mutant (Fig. 5A), indicating increased podosome-nucleating activity. Quantification of the podosome F-actin intensity in cells stained with fluorescently labelled phalloidin revealed that Y291E podosomes had slightly less F-actin, whereas the area covered by the F-actin signal was also smaller in Y291E cells compared with control and Y291F cells (Fig. 5B,C). However, when normalised for podosome area, the Y291E cells had a higher
F‐actin content than podosomes of wt or Y291F cells. Rotary shadowing electron microscopy was performed to determine whether this difference in F‐actin content in Y291E mutant cells resulted in altered podosome ultrastructure. Confirming our immunofluorescence finding, the WASp Y291E‐induced podosomes were smaller in size but appeared to be more three dimensional relative to control and Y291F‐expressing cells (Fig. 5E).

WASp Y291 phosphorylation affects podosome assembly rates and actin dynamics

In vitro studies have suggested a role for (N)WASp phosphorylation in the increase in the rate of Arp2/3‐dependent actin polymerisation (Suetsugu et al., 2002; Torres and Rosen, 2003). Actin dynamics of podosomes were therefore studied using photobleaching experiments. To obtain a measure of the rate of actin polymerisation inside individual podosomes, FRAP (fluorescence recovery after photobleaching) was performed in cells expressing EGFP‐β‐actin by bleaching an area containing podosomes and following the fluorescence intensity recovery in pre‐existing podosomes. Surprisingly, rates of actin recovery in podosomes were unaltered in control and Y291 mutant cells, with a half‐life (t1/2) of approximately 6 seconds for all cells, even though the Y291E‐expressing cells showed an increased, albeit insignificant, immobile fraction compared with both control and Y291F‐expressing cells (supplementary material Fig. S1A). Therefore, in pre‐existing podosomes, the rate of actin recovery does not depend on the phosphorylation status of WASp. Phosphorylation does, however, affect the rate of nucleation of nascent podosomes as determined by podosome reassembly experiments (Fig. 6A). In these, macrophages plated on fibronectin‐coated coverslips were treated with cytochalasin D, which completely dissolved all podosomes, and the synchronous formation of new podosomes was initiated by removal of cytochalasin D. Faster recovery of the number of cells displaying podosomes was observed for the Y291E mutant, which was almost complete by 10 minutes. Furthermore, the number of podosomes per cell was significantly higher for control and Y291E cells than in the Y291F cells (Fig. 6B; supplementary material Fig. S2). Therefore, Y291 phosphorylation appears to be important for the efficient nucleation of nascent podosomes rather than the rate of actin polymerisation.

Inversely, FLIP (fluorescent loss in photobleaching) was used as a method to examine the lifetime of actin inside podosomes and, consequently, obtain a measure of actin disassembly. The data obtained indicated that GFP‐β‐actin had a longer half‐life inside podosomes of cells expressing the Y291E mutant, compared with control and Y291F‐expressing cells (Fig. 6C; supplementary material Movies 1‐3). These data indicate an enhanced stability of F‐actin and are consistent with the apparently longer filaments observed in Y291E cells and cells expressing the Y291E/F point mutants (Fig. 6D).
To determine whether the observed differences in podosome and chemotaxis were related to altered rates of WASp exchange but might instead be due to alterations in actin and podosome dynamics, therefore, not due to altered rates of WASp exchange but might instead be due to differences in their activities.

To determine whether WASp activity was reduced in cells unable to phosphorylate WASp, TIRF/FRET microscopy was performed with cells expressing a phospho-deficient form of the WASp biosensor. Surprisingly, the WASp Y291F biosensor was as equally active in podosomes as the wild-type biosensor (Fig. 6E). However, unlike the restriction of the active wild-type WASp biosensor to podosomes, WASp Y291F activity was detected all along the ventral surface of the cell (compare Fig. 6F with Fig. 1B). Nevertheless, no differences could be observed in the overall localisation of the wild-type or Y291F biosensors either by TIRF or epifluorescence microscopy (supplementary material Fig. S3) suggesting it is only the active Y291F species that is mislocalised. This data suggests that Y291 phosphorylation could be required for the restriction of active WASp in podosomes.

WASp Y291 phosphorylation is required for matrix degradation and chemotaxis.

To determine whether the observed differences in podosome dynamics resulted in functional consequences, the ability of the H246D-, Y291E- or Y291F-expressing cells to degrade fibronectin matrices was also assessed. As shown in Fig. 7A,B the Y291E clone could degrade fibronectin as efficiently as the control cells; the H246D cells only had minimal proteolytic activity, which was comparable with the shWASp cells, whereas the Y291F cells showed significantly reduced proteolysis. Additionally, the Y291E cells had better defined podosomes than Y291F cells, and were able to extend into a thick and more pliable matrix of gelatin-agarose overlaid by fibronectin (supplementary material Fig. S4). Also, as with the thin matrix, degradation of fibronectin overlaid on gelatin-agarose resulted in a similar pattern in control, Y291E and Y291F cells (data not shown).

The differences in proteolytic activity observed were linked to the ability of cells to form podosomes and not to adhesion defects because all of the rescue cell types were able to adhere equally to fibronectin and vitronectin substrates (supplementary material Fig. S5). When plated on fibronectin, Y291F expression resulted in diminution of both the number of cells with podosomes and the number of podosomes per cell compared with levels obtained when cells were plated on uncoated surfaces in the presence of serum (Fig. 7C,D; compare with Fig. 4B and Fig. 5A). However, serum starvation of cells plated on fibronectin resulted in all cells forming podosomes equally (see Fig. 6A; supplementary material Fig. S2). Expression of the Y291E construct resulted in slightly, yet significantly, more podosomes per cell compared with the other clones, whether cells were plated directly on glass or fibronectin-coated coverslips (Fig. 7D, compare with Fig. 5A).

Nevertheless, defects associated with adhesion were observed because the H246D-expressing cells and to a lesser extent, the Y291F-expressing cells, spread more slowly on fibronectin substrata. Interestingly, this spreading defect was not observed on vitronectin matrix. Furthermore, fewer Y291F-expressing cells displayed podosomes than did control and Y291E-expressing cells when plated on fibronectin, but not on vitronectin, under the same conditions (data not shown). Our data therefore, indicate a distinct behaviour of the cells expressing the two different Y291 mutations, when these are plated on glass or fibronectin matrices. Tyrosine
phosphorylation is therefore important for the formation of well-defined podosomes on fibronectin matrices as opposed to uncoated or vitronectin-coated surfaces.

Podosomes have been linked to the ability of macrophages to perform chemotaxis. Since the Y291 point mutant rescue cells had podosomes, we hypothesised that they would still be able to chemotax in a Boyden chamber assay in response to CSF-1. Surprisingly, Transwell assays revealed that neither the Y291F nor the Y291E point mutant could rescue the chemotaxis defect associated with WASp silencing (Fig. 7E), even though the basal migration was similar in all the cells tested. As expected, the H246D mutant was also unable to chemotax. These results reinforce the central role of Cdc42 in WASp function in macrophages, but point to more complex roles for tyrosine phosphorylation in the regulation of chemotaxis, suggesting that both phosphorylation and dephosphorylation mechanisms are necessary for chemotaxis.

Discussion

WASP is a haematopoietic-specific actin nucleator that acts via the Arp2/3 complex to polymerise actin filaments. In this study, through the use of RNA interference and rescue approaches, we sought to investigate the regulatory mechanisms of WASp in podosome formation and function. WASp is a Cdc42 effector, typified by the presence of a GBD. Our data point to an absolute requirement for Cdc42 binding in podosome formation, as evidenced by using a Cdc42-binding-defective mutant WASp and Cdc42 shRNA in macrophages. Interestingly, the H246D point mutation significantly reduces pervanadate-induced tyrosine phosphorylation of a WASp biosensor (Cammer et al., 2009), supporting the allosteric regulation model (Torres and Rosen, 2003; Torres and Rosen, 2006), whereby WASp phosphorylation is dependent on unfolding by Cdc42. Nevertheless, Cdc42-independent mechanisms of (N)WASp activation and phosphorylation have also been described (Badour et al., 2004; Campellone et al., 2008; Padrick et al., 2008; Rivera et al., 2004; Sallee et al., 2008; Tomasevic et al., 2007; Yarar et al., 2007).

An intriguing finding comes from the fact that WASP Y291 phosphorylation is implicated in podosome formation depending on the context of the matrix and its physical properties. It is dispensable for podosome formation when macrophages are plated directly on glass (Fig. 4) or vitronectin (not shown) but is required for efficient podosome formation on fibronectin matrices. This raises...
the possibility that WASp can transduce signals from integrins, a hypothesis that is supported by the fact that integrin clustering can lead to WASp phosphorylation (Chellaiah et al., 2007; Gismondi et al., 2004; Lutskiy et al., 2007; Soriani et al., 2006).

WASp activity was also required for efficient fibronectin degradation, indicating that podosomes act as scaffolds for proteolytic activity. However, whether or not WASp has a direct role in the trafficking and release of MMPs to podosomes or an indirect role, through the formation of podosomes, is unknown. Reflecting the ability of cells to form podosomes on fibronectin, the extent of fibronectin matrix degradation was also dependent on WASp Y291 phosphorylation. Hence, the diminished ability of the Y291F mutants to form podosomes on fibronectin resulted in diminished fibronectin proteolysis. WASp tyrosine phosphorylation could be an important signal downstream of fibronectin receptors to sense matrix pliability and signal its degradation via podosome formation and MMP targeting.

An unexpected finding comes from the discovery that, in intact cells, WASp tyrosine phosphorylation does not appear to further enhance the rate of actin polymerisation in existing podosomes, as determined by FRAP. By contrast, tyrosine phosphorylation of WASp is important for the accelerated formation of nascent podosomes, as well as the formation of numerous podosome cores. F-actin undergoes a few cycles of depolymerisation and repolymerisation during the lifetime of a podosome, and modelling has suggested that a filament-severing mechanism is responsible for depolymerisation (Destaing et al., 2003), for example by gelsolin or cofilin. The depolymerisation rates, a measure of which can be provided by FLIP, however, would suggest that severing may be reduced in the Y291E mutant. This would imply that fewer barbed ends would be formed for repolymerisation to occur, which would, in turn, explain both the diminished size of the Y291E podosomes compared with Y291F podosomes, as well as the increased F-actin content per area. Additionally, this might provide a means for the generation of longer podosomes, and could explain the greater extent of podosome insertion into a thick matrix by control and WASp Y291E-expressing cells. This mechanism is also supported by the FRAP data, which suggest a larger immobile fraction of actin in the Y291E cells compared with levels in control cells. Although this increase was not significant, this trend was not observed in the Y291F cells. Further studies are required to identify how WASp contributes to filament disassembly and to address the apparent discrepancy between the FRAP and FLIP data.

The underlying mechanism for the effects observed by the phospho-abolishing WASp mutant could, at least in part, be due to inefficient localisation or residence of the protein in podosomes. Indeed, our results using a WASp biosensor coupled to TIRF microscopy, suggest that WASp activity in the Y291F point mutant is diffusely localised along the ventral surface of the macrophage. Notably, the Y291F WASp biosensor was not any less active compared with the wild-type biosensor in podosomes. Ineffective targeting might therefore explain the inefficient nucleation of podosomes and the instability of F-actin, as observed by FLIP and wiskostatin-induced podosome disassembly. Nevertheless, FRAP and FLIP experiments of GFP-WASp revealed equal rates of exchange in podosomes between wild-type and phospho-mutant proteins, even though this approach cannot discriminate between active and inactive species. Therefore, the observed differences in podosome formation and dynamics are more likely to be due to distinct functions of the phospho-abolishing or phospho-mimicking mutants rather than altered kinetics of association with podosomes.

Enhanced actin polymerisation might not account for a direct output of WASp activity in vivo, as in vitro studies suggested, but instead the resulting stability of filaments might be a more accurate

**Fig. 7.** WASp tyrosine phosphorylation is required for podosome formation and function on a fibronectin matrix. (A) The indicated cells were plated on an Alexa Fluor 488-labeled fibronectin matrix and stained for F-actin. White arrowheads indicate examples of areas with degraded fibronectin. Scale bar: 20 μm. (B) Fibronectin proteolysis was expressed as the degradation area per cell. (C) Decreased podosome formation in the Y291F-expressing cells on fibronectin. (D) WASp tyrosine phosphorylation affects podosome number in macrophages plated on fibronectin (control, Y291E and Y291F, n=40 cells; shWASp, n=20 cells; H246D, n=15 cells from a representative experiment). (E) Chemotaxis towards CSF-1 requires Cdc42 binding and tyrosine phosphorylation-dephosphorylation of WASp. Chemotactic ability of the indicated cells was determined using a modified Transwell chamber assay. Cells were placed into the chamber of a Transwell insert in the absence (RPMI; white bars) or presence of 20 ng/ml CSF-1 (black bars) in the lower chamber. After 4 hours, chemotaxis was determined by counting the cells in nine different fields. *P<0.05; **P<0.01 relative to control.
description of the outcome of WASp activity, because WASp might influence filament capping and severing (Weisswange et al., 2009). Interestingly, a recent study revealed that the activating L229P and L232P mutations in N-WASP, although they enhanced actin polymerisation in pyrene assays, resulted in shorter actin tails being formed beneath Shigella in infected cells, suggesting a more pronounced role of activated N-WASP on actin depolymerisation in vivo (Adamovich et al., 2009).

The inability of WAS monocytes and macrophages to chemotax has been linked to their inability to polarise (Badolato et al., 1998), to correctly localise the Arp2/3 complex and to form podosomes (Linder et al., 2000; Zicha et al., 1998). Podosomes are commonly formed at the leading edge of a migrating cell, thereby defining the direction of motility, and WAS leukocytes can still move, although not directionally (Jones et al., 2002; Zicha et al., 1998). Podosomes could act in directional migration by stabilising protrusions through increased adhesion at the leading edge and the interplay between adhesion and de-adhesion via podosome formation and disassembly or altered actin dynamics might allow directional persistence and forward translocation of the cell. Our data support such a dynamic relationship between podosomes and directional migration. Indeed, the Y291F and Y291E mutants, although capable of assembling podosomes, were not able to chemotax. This would suggest that phosphorylation-dephosphorylation cycling is required for efficient chemotaxis, potentially as a mechanism to modulate actin dynamics of podosomes by stabilising (WASp phosphorylation) or weakening (WASp dephosphorylation) actin filaments. Indeed, recent reports have linked increased podosome turnover or disassembly to enhanced motility in DCs (Calle et al., 2006; van Helden et al., 2006), whereas, inversely, stasis of macrophages has been correlated with increased podosome formation and gelatinolytic activity (Perri et al., 2007). WASp tyrosine phosphorylation and dephosphorylation could provide a way to regulate motility through the regulation of podosome turnover rates.

In conclusion, the results of this study point to a more complex role for tyrosine phosphorylation than simply in the regulation of WASp activity, and suggest a link between podosome dynamics and macrophage migration. Additional studies will be needed to determine the mechanism by which phosphorylation of WASp regulates its function.

Materials and Methods

Cells, antibodies and reagents

RAW/LR5 cells were derived from the murine monocye-macrophage RAW 264.7 cell line and were grown in RPMI medium (Mediatech), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Human monocyte-derived macrophages were a gift from Indra Sethy-Coraci (Columbia University, NY). All cells were maintained at 37°C in a 5% CO₂ incubator. Recombinant mouse CSF-1 was purchased from R&D Systems (Minneapolis, MN). Purified human plasma fibronectin was from Sigma, whereas vitronectin was from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibodies against WASp (H-250) and Cdc42 (P1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal vinculin (hVIN-1) and β-actin (AC-15) antibodies were from Sigma. Mouse monoclonal Myc-tag antibody (9B11) was from Cell Signaling Technology (Beverly, MA). Alexa Fluor 568-phalloidin and secondary antibodies conjugated to Alexa Fluor 488 or 568 used for immunostaining were from Molecular Technology (Beverly, MA). Alexa Fluor 568-phalloidin and secondary antibodies conjugated to Alexa Fluor 488-labelled goat anti-mouse IgG. Podosomes were considered as such when a vinculin ring surrounded F-actin punctate structures organised in clusters.

Immunofluorescence microscopy and morphological analysis

Cells plated on 12 mm glass coverslips (uncoated or coated with fibronectin as indicated in the text) were fixed in 3% formaldehyde in BWD buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 5 mM glucose, 10 mM NaHCO₃, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.4) for 7 minutes, permeabilised in 0.2% Triton X-100 (in BWD) for 5 minutes, and blocked with 10% milk in TBS. F-actin was detected using a specific monoclonal antibody (1:200 dilution) followed by incubation with Alexa Fluor 488-labelled goat anti-mouse IgG. Podosomes were considered as such when a vinculin ring surrounded F-actin punctate structures organised in clusters. At least 60 cells per experiment were analysed for each determination, unless otherwise indicated. Images were taken using the ×60/1.4 NA phase objective of an Olympus IX71 microscope coupled to a Sensicam cooled CCD camera. Confocal images were acquired on a Zeiss 5 Live Duoscan confocal microscope using a Plan Apo 63/1.40 NA oil DICI objective. Image J 1.41f was used to quantify podosome F-actin intensity and size.

Plasmin and site-directed mutagenesis

The WASp biosensor is described elsewhere (Cammer et al., 2009). Point mutations were introduced in the human WASp cDNA sequence cloned in the pE8-BOS vector or the biosensor plasmid using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The following mutagenic primers were used (all primers in 5’ to 3’ direction; underlined nucleotides denote the mutated codon): H246D: CA ACC AGT GGA TTC AAG GAT GTC AGC CAC GTG GGG; Y291F: G ACC TCT AAA CTT ATC GTC CAC TTC ATT GAC GAC C; Y291E: G ACC TCT AAA CTT ATC GTC CAC TTC ATT GAC GAC C; the WASp cDNA was cloned to the pEGFP-C1 plasmid (Invitrogen) from the biosensor plasmid using HindIII-BamHI restriction sites.

Stable transfection

The human WASp sequence differs by four nucleotides from its murine counterpart in the region targeted by the shRNA and therefore was not recognised by the silencing machinery. RAW/LR5 cell transfections were performed using the SuperFect reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Subconfluent cells in 6 cm dishes were incubated with a transfection mix containing 10 µg of the Myc-WASP expressing plasmid and psRt (10:1,w:w) and 20 µl SuperFect for 6 hours at 37°C. The transfection mix was then replaced by fresh complete medium. After 24 hours, cells were lifted and replated in 24-well plates in the presence of G418 (Invitrogen; resistance conferred by the psRt plasmid). Single-cell-derived colonies were isolated and expanded in culture. Stable expression of the protein of interest was confirmed by western blotting.

RNA-mediated interfeference

Reduction of WASp expression in RAW/LR5 cells was performed using the psiSUPER RNAi system from Oligoengine (Seattle, WA), according to the manufacturer’s instructions. An oligonucleotide corresponding to the 363-381 portion of the open reading frame of mouse WASp cDNA was used as a target sequence (5’-AGATGACTGTCAGATAGGAGA-3’). The insert encoding the short hairpin RNA (shRNA) against WASp transcript was cloned between the BglII and HindIII restriction sites of the psiSUPER retro.puro vector. The resulting plasmid was transfected into a HEK293T-based packaging cell line and the cell culture supernatant was used to retrovirally infect RAW/LR5 cells. Stable mass population with reduced WASp expression was obtained after puromycin selection (resistance brought by the psiSUPER retro.puro plasmid) of the infected cells. Control cells were generated by retroviral infection of a psiSUPER retro.puro plasmid coding for a control non-silencing shRNA sequence (5’-TCCACACACTACAAGGAC-3’). Cells with normal or reduced WASp expression showed no difference in their WAVE2 protein levels, the other member of the WASp family predominantly found in macrophages (not shown). Also, no difference in cell viability was observed between cells with normal or reduced WASp expression as assessed by Trypan blue exclusion (at least 95% viable cells in all conditions). The sequences used for the psiCD42 shRNA are described elsewhere (Park and Cox, 2009).

Western blotting

Cells were lysed in ice-cold buffer A containing 25 mM Tris-HCl, 137 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM orthovanadate, 1 mM benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin, pH 7.4. Whole-cell lysates were resolved by SDS-PAGE and proteins were transferred onto PVDF membranes (Immobilon-P, Millipore) that were subsequently blocked using 5% BSA, 1% ovalbumin in TBS containing 0.1% Tween20 before incubation with primary antibodies overnight at 4°C. Membranes were then washed and incubated with secondary antibodies conjugated to HRP. Signals were visualised using the SuperSignal West Pico Chemiluminescent Substrate from Pierce and images were acquired using a Kodak Image Station 440.

Transmigration assay

Cell chemotaxis was measured using a transmigration chamber assay with 8 µm pore size inserts (Falcon) according to the manufacturer’s instructions. Briefly, the inserts were placed into 24-well plates containing RPMI in the presence or absence of 20 ng/ml CSF-1. 500,000 serum-starved cells were then loaded onto the inserts and incubated at 37°C for 4 hours. Cell migration was quantified by counting the number of cells that migrated through the insert (nine different fields in each well were counted under a phase-contrast microscope, using a ×4 objective) and was expressed as the average number of cells per field of view or fold induction of migrated cells in the absence of chemotaxin.
Matrix degradation assay

The ability of RAW/LR5 cells to degrade fibronectin was assessed by plating cells onto fluorescently labelled fibronectin films on top of gelatin (Yamaguchi et al., 2005) (supplementary material Fig. S4) or poly-L-lysine (Fig. 7) and incubated in complete medium for 16 hours at 37°C. Matrix degradation activity was indicated by local losses of fluorescence of fibronectin. Areas of degraded matrix were then traced and measured in at least 15 different fields per experiment and expressed as percentage of control cells (supplementary material Fig. S4), or degradation area per cell (Fig. 7).

Adhesion assays

For adhesion assays, glass coverslips were coated overnight at 4°C with fibronectin (10 µg/ml) or vitronectin (2 µg/ml). Cells were serum starved in suspension in RPMI for 2 hours and coverslips were washed three times with PBS in 24-well dishes and 9×10^4 cells per well were seeded. Cells were fixed, 1, 2 or 3 hours after plating and stained with phalloidin, vinculin and DAPI. For determination of number of adherent cells, at least seven random fields were viewed using the ×20 objective, while for morphological analyses images were taken from five random fields using the ×60 objective of an Olympus IX71 microscope (see above).

FRET and TIRF microscopy

The WASp FRET-based biosensor has been described and characterised elsewhere (Cammer et al., 2009). Briefly, the biosensor is based on the previously described N-WASP biosensor (Lorenz et al., 2004) that exhibits intramolecular FRET between a cyan fluorescent protein and a yellow fluorescent protein fused to the N- and C-termini respectively, when the molecule is in an autoinhibitory conformation. RAW/LR5 cells were transfected with 1 µg of the WASp biosensor plasmids on six-well plates and seeded after 24 hours onto glass bottom dishes (MatTek Corporation). FRET images were acquired with an automated Olympus IX70 microscope equipped with filter wheels in the excitation and emission light path and coupled to a cooled SensiCam QE CCD camera (Cooke, Romulus, MI). CFP was excited using a S430/25 filter with a Sutter DG4 illuminator (Sutter Instruments, Novato, CA) and the fluorescence detected with a S470/30 (donor image) or S535/30 (FRET image) emission filter. YFP was imaged with exciter S500/20 and emitter S535/30 (YFP image). In all cases a dual-band dichroic mirror 86002v2bs was used (Chroma Technology). To examine a role for WASp in the stabilisation or adherence of protrusions and in the formation of podosomes, we examined WASp-biosensor expressing cells using total internal reflection microscopy (TIRF, Olympus objective; ×60/NA 1.45 lens). The activation of WASp was detected by dividing the donor image (CFP excitation-CFP emission) by the FRET image (CFP excitation-YFP emission). Acquisition and FRET analyses were done with IP Lab v3.51 (Scanalytics, VA). For comparison between podosome versus non-podosome areas, more than 200 podosome from a total of 11 cells were analysed, and an equal number of randomly selected ventral, non-podosomal areas of similar size to the podosomes were selected and analysed as described above. In all cases, cells expressing equal levels of the WASp biosensor were selected for analysis.

Photobleaching experiments

FLIP and FRAP were performed on a Zeiss 5 Live DuoScan confocal microscope on a heated stage and chamber (37°C), with 2% laser power (488 nm) and an open pinhole. Cells transiently transfected with the appropriate plasmids (pCDNA3/EGFP-β-actin, a Plan b-actin, a Plan 63/1.4 NA oil DICI objective was used. For FRAP, images were acquired at 10 frames/second and after 10 frames a region (5×11 pixels) was photobleached using a single pulse of six iterations at 100% laser power. Typically, this would cover an area of 5-8 podosomes, which were photobleached simultaneously. Imaging was allowed to proceed for 360-420 frames.

Movies of cells were imported using ImageJ 1.41f and plots of fluorescence recovery (FRAP) or loss (FLIP) from podosomes were transferred in Microsoft Excel. The number of cells analysed per condition are indicated in the respective figure legends, with approximately five podosomes could be analysed for FRAP and ten or more podosomes per cell were analysed for FLIP measurements. All plots were normalised to background and bleaching, whereas time was adjusted to delays due to the bleach cycles. The obtained curves were fitted to single exponentials using Matlab and the half-lives determined.

Podosome disassembly and reformation assay

Cells were plated over fibronectin films and subsequently serum-starved for 4 hours. For disassembly experiments, wiskostatin was added for the indicated time points at a concentration of 1.5-5 µM, and cells were fixed and stained for podosome visualisation. For reformation assays, cytochalasin D was added to the medium and cells were incubated for 10-15 minutes, resulting in complete dissolution of podosomes. Cytochalasin-D-containing medium was removed and replaced with warm serum-free RPMI supplemented with 25 nM HEPES, pH 7.4 for the indicated times. Cells were fixed, permeabilised and stained for F-actin and vinculin, and podosomes were scored as described above.

Electron microscopy

Macrophages plated on 5 mm glass coverslips were perfused with 2 minutes at 37°C in cytosekeletal buffer (5 mM KCl, 137 mM NaCl, 4 mM NaHCO3, 0.4 mM KH2PO4, 1.1 mM Na2HPO4, 2 mM MgCl2, 5 mM PIPES, 2 mM EGTA, 5.5 mM glucose, pH 6.0), supplemented with 0.75% Triton X-100 and 5 µM phallolidin. Subsequently, cells were fixed in cytosekeletal buffer containing 1% glutaraldehyde and 5 µM phallolidin for 15 minutes, after which cells were rinsed three times in distilled water, flash frozen and prepared for rapid freezing, freeze-drying and rotary shadowing as described (Baily et al., 1999). The samples were observed using a transmission electron microscope (100CX; JEOL) at 100 kV. By convention, images were viewed as negatives.

Data and statistical analyses

In all cases data are expressed as the mean ± s.e.m. from a minimum of three independent experiments, unless otherwise indicated. Statistical significance of the data was determined using the two-tailed Student’s t-test. P<0.05 was considered significant.

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