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Microtubule-dependent formation of podosomal adhesion structures in primary human macrophages

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SUMMARY

Podosomes are unique actin-rich adhesion structures of monocyte-derived cells such as macrophages and osteoclasts. They clearly differ from other substratum-contacting organelles like focal adhesions in morphological and functional regards. Formation of podosomes has been shown to be dependent on the small GTPase CDC42Hs and its effector Wiskott-Aldrich syndrome protein (WASp). In this study, we investigated the functional relation between podosomes and the microtubule system in primary human macrophages. We demonstrate that, in contrast to focal adhesions, assembly of podosomes in macrophages and their monocytic precursors is dependent on an intact microtubule system. In contrast, experiments using Wiskott-Aldrich syndrome (WAS) macrophages indicate that the microtubule system is not reciprocally dependent on podosomes. A potential linker between podosomes and microtubules may be WASp itself, considering that microinjection of the WASp polyproline domain prevents podosome reassembly. This polyproline domain is thought to link WASp to microtubules via CDC42 interacting protein 4 (CIP4). Consistently, macrophages microinjected with CIP4 constructs deficient in either the microtubule- or the WASp-binding domain also fail to reassemble podosomes. In sum, our findings show that microtubules are essential for podosome formation in primary human macrophages and that WASp and CIP4 may be involved in this phenomenon.

Key words: Podosome, Microtubule, Primary macrophage, WASp, CIP4

INTRODUCTION

Cells of the monocytic lineage like primary macrophages and osteoclasts form unique and highly dynamic adhesion structures called podosomes (Marchisio et al., 1984; Marchisio et al., 1987; Zambonin-Zallone et al., 1989). Podosomes are sites of extravasation of the plasma membrane (Tarone et al., 1985) that contact the substratum via integrins (Marchisio et al., 1988; Gaidano et al., 1990). They consist of a core of short actin filaments and actin-associated proteins which is surrounded by a ring of vinculin and talin (Gavazzi et al., 1989; Zambonin-Zallone et al., 1989). Recently, the small GTPase CDC42Hs and its effector Wiskott-Aldrich syndrome protein (WASp) have been shown to control podosomes in primary human macrophages (Linder et al., 1999). A crucial WASp-regulated step in this process is the recruitment of the Arp2/3 complex to sites of podosome formation (Linder et al., 2000). Absence of podosomes and the concomitant defects in macrophage adhesion and polarization probably contribute to the severe immunodeficiency observed in patients suffering from Wiskott-Aldrich syndrome (WAS), a disease caused by mutations in WASp (reviewed by Ochs, 1998).

Podosome formation can also be induced in fibroblasts upon transformation with viruses whose oncogenes code for protein tyrosine kinases (Tarone et al., 1985). In turn, podosomes in primary human macrophages can be destroyed by use of tyrosine kinase inhibitors (S. Linder et al., unpublished observation). This need for tyrosine phosphorylation in podosome genesis and maintainence is shared by other adhesion structures like focal adhesions (Burridge et al., 1992; Romer et al., 1994). Podosomes and focal adhesions have other elements in common like vinculin and talin. However, they clearly differ from each other in many important regards (cellular distribution, overall architecture and other characteristics) as (1) actin is an integral protein of podosomes but not of focal adhesions, (2) proteins which control actin assembly like CDC42Hs, WASp and Arp2/3 complex are present in podosomes (Linder et al., 1999; Linder et al., 2000) but not in focal adhesions (S. Linder et al., unpublished), (3) podosome formation does not require de novo protein synthesis like formation of focal adhesions (Tarone et al., 1985), (4) podosomes in Rous sarcoma virus-transformed BHK fibroblasts form during the initial adhesion phase within 1 hour while focal adhesion assembly requires a minimum of 3 hours (Tarone et al., 1985).

Podosomes are the most prominent part of the actin

cytoskeleton of primary human macrophages. In this study, we investigated the functional relationship of podosomes and another part of the cytoskeleton, the microtubule system. Interactions between the actin and microtubule cytoskeletons are numerous (reviewed by Goode et al., 2000) and the influence of microtubules on adhesion structures like focal adhesions is well documented (Kaverina et al., 1998; Kaverina et al., 1999). Microtubules are necessary for the dissociation of focal adhesions and, in turn, microtubule depolymerization by drugs like nocodazole or vinblastine leads to greatly enhanced focal adhesion formation (Bershadsky et al., 1996). In contrast, there are at present no data available concerning the role of microtubules in podosome turnover. An influence of microtubules on podosome positioning, however, has been described (Babb et al., 1997).

In the present study we show that microtubules are required for podosome formation whereas, vive versa, loss of podosomes does not affect the architecture of the microtubular network. We further show that macrophages microinjected with the isolated WASp polyproline domain, which is thought to link WASp to microtubules via CDC42 interacting protein 4 (CIP4; Aspenström, 1997; Tian et al., 2000) fail to reassemble podosomes. Additionally, microinjection of macrophages with CIP4 deletion mutants lacking either the microtubule-binding Fer and Fes/Fps homology domain (FCH) or the WASp-binding Src homology 3 domain (SH3) also leads to a failure in podosome reassembly. These data indicate that formation of macrophage podosomes crucially depends on microtubules and that WASp and CIP4 may be involved in this functional dependency.

MATERIALS AND METHODS

Cell isolation and cell culture

Human peripheral blood leukocytes were isolated by centrifugation of heparinized blood in Ficoll (Biochrom, Berlin, Germany). Monocytic cells were isolated with magnetic anti-CD14 antibody beads and an MS+ separation column (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions and seeded onto Cellocate coverslips (Eppendorf, Hamburg, Germany) at a density of 5×10^4. Cells were cultured in RPMI containing 20% autologous serum at 37°C, 5% CO_2 and 90% humidity. Medium was changed every 3-4 days.

WAS macrophages

Patient 3,603 (Schindelhauer et al., 1996) shows the mutation C995T, leading to a premature stop codon and expression of a C-terminally truncated form of WASp. Mononuclear cells were isolated from peripheral blood and cultured like cells from healthy donors. WAS cells were used with the explicit permission of the patient.

Adhesion experiments

Monocytes were eluted from the separation column (see Cell Isolation) and washed once in cold RPMI. Cells were then seeded onto glass coverslips at a density of 5×10^4 either immediately or after addition of nocodazole (1 μM in DMSO), benomyl (100 μM in DMSO) or vinblastine (1 μM in DMSO), respectively (all Sigma, Deisenhofen, Germany). Cells were allowed to adhere for the indicated times. For wash out of drugs, coverslips were placed 2 times for 5 minutes in RPMI (37°C) and then in fresh RPMI for an additional 2 hours. Monocytes in control experiments were treated with DMSO alone and developed normal microtubules and podosomes, comparable to untreated cells.

Podosome disruption experiments

Macrophages cultured for 6 days on coverslips were placed into wells of 24-well plates (Nunc, Wiesbaden, Germany) containing 2 ml RPMI medium with 2 μM cytochalasin days (Sigma) or 25 μM tyrosine kinase inhibitor PP2 (Sigma) for 1 hour. Coverslips with macrophages were either directly fixed or washed free from drugs. For wash out, coverslips were transferred to new wells containing fresh RPMI medium, incubated for 2 minutes, again transferred into fresh RPMI and finally incubated in fresh RPMI for the times indicated. Wash-out buffer was supplemented with 1 μM nocodazole and/or 1 mM RGDS peptide (both Sigma) as indicated.

Generation of WASp constructs and protein expression

WASp polyproline construct (aa311-aa404) was created by cloning a PCR-generated insert into the *Eco*RI and *Bam*HI sites of vector pGEX-2T (Pharmacia, Uppsala, Sweden). WASp verprolin-like/central ('VC') and acidic constructs ('A') were created as described earlier (Linder et al., 1999; Linder et al., 2000). Dynamin II polyproline construct (Dong et al., 2000) was a kind gift of Dr Peter Westermann (Max-Delbrück Centrum, Berlin, Germany). Aa741-aa870 of dynamin II were expressed via vector pET30a(+) as a His- and S-peptide-tagged fusion protein in *E. coli* and purified on Ni-NTA and Superdex 75 columns. CIP4 deletion constructs allowing expression of aa1-481 and 118-545 of CIP4, respectively, as GST-fusion proteins (Tian et al., 2000) were kind gifts from Dr Donn M. Stewart (NIH, Bethesda, USA). Proteins were dialyzed against microinjection buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$), concentrated in Centricon filters (Millipore, Bedford, USA), shock-frozen and stored at –80°C. Purity was tested by SDS-PAGE and Coomassie staining.

Immunofluorescence microscopy

For tubulin staining, cells were lysed in 50% PHEM-buffer (20 mM HEPES, 4 mM EGTA, 1 mM $MgCl_2$) with 1 mM vanadate, 5% hexyleneglycol and 1% Brij 58 at room temperature for 1 minute, washed briefly in 50% PHEM, fixed in 50% PHEM with 1% glutaraldehyde for 10 minutes and permeabilized in ice-cold acetone for 5 minutes (modified from Koonce et al., 1986; all reagents Sigma), except for WASp/tubulin costainings (Fig. 9) which were performed as described (Tian et al., 2000). Briefly, cells were lysed and fixed in 4% formaldehyde, 0.1% Triton X-100, 80 mM PIPES, pH 7.4, 1 mM EGTA, 1 mM $MgSO_4$, 30% glycerol at room temperature for 30 minutes. Background fluorescence was reduced by incubating coverslips with 5% human serum and 5% normal goat serum (both Dianova, Hamburg, Germany) in PBS. Tubulin was stained with monoclonal mouse anti-α-tubulin clone B 512 or with monoclonal mouse anti-β-tubulin clone 2.1 antibody (both Sigma) as indicated, except for WASp/tubulin costainings where monoclonal rat anti-α tubulin antibody (Serotec, Oxford, UK) was used. No difference in tubulin staining using either antibody was observed. WASp was stained using monoclonal antibody 3D8.H5 (Stewart et al., 1996). For actin staining, cells were fixed for 10 minutes in 3.7% formaldehyde solution and permeabilized for 10 minutes in ice-cold acetone. Actin was stained with Alexa 568-labeled phalloidin (Molecular Probes, Leiden, Netherlands), and vinculin was stained with monoclonal anti-vinculin antibody (Sigma). Secondary antibodies were Alexa 488- and 568-labeled goat anti-mouse (Molecular Probes) or FITC labeled goat anti-rat IgG antibody (Dianova). Coverslips were mounted in Moviol (Calbiochem, Schwalbach, Germany), containing *p*-phenylendiamine (Sigma) as anti-fading reagent, and sealed with nail polish. Images were obtained either through single section confocal laser scanning microscopy (Leica, Wetzlar, Germany) or with a spot digital camera (Leica). Laser scanning confocal micrographs of tubulin in Figs 1, 3, 6, 8, 10 were obtained by superimposition of each time 2-4 individual optical slices with a scanning difference of 0.2-1.0 μm.

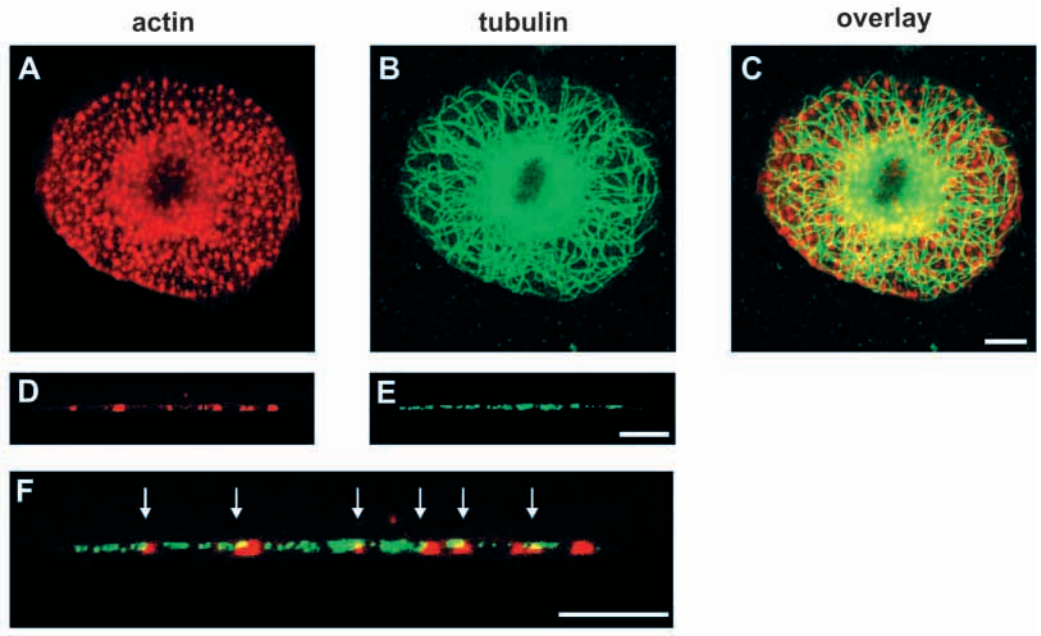

Fig. 1. Actin and microtubule cytoskeleton of a primary human macrophage, 6 days old. Confocal laser scanning micrographs showing horizontal sections of ventral parts of cell (A-C) or vertical sections of peripheral parts of cell (D-E), f-actin stained with rhodamine-phalloidin, red color (A,D), tubulin stained with anti-α-tubulin antibody, green color (B,E), overlay of A and B (C), and of D and E (F), yellow color indicates colocalization. Microtubules appear to be closely associated with podosomes and, in some cases, to directly contact them. (Note: MTOC is out of focal plane in B and therefore not visible). Bars, 10 μm, for all panels of the same row.

GST pull-down assay and immunoblotting

GST pull-downs were prepared as described previously (Linder et al., 2000). Briefly, $6x10^6$ cells cultured for 6 days were lysed by addition of 200 μl/well of RIPA buffer (10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 140 mM NaCl) containing protease inhibitors. After centrifugation (15,000 rpm, 15 minutes, 4°C), aliquots of the supernatant were added to glutathione Sepharose beads, previously incubated for 1 hour with 100 μg of GST-fusion proteins. Beads were incubated with lysate for 2 hours at 4°C, washed 2 times for 1 minute and once for 10 minutes, pelleted, 100 μl of SDS-sample buffer was added and an aliquot was run on a 12.5% SDS gel. Western blots were prepared as described previously (Linder et al., 1999). Arp2/3 p34-Arc and p41-Arc were detected by using polyclonal antibodies (Linder et al., 2000). No binding of p34-Arc or p41-Arc was detected when GST alone was bound to beads.

Microinjection of proteins

Cells for microinjection experiments were cultured for 5-8 days. Microinjection was performed using transjector 5246 (Eppendorf) and a Compic Inject micromanipulator (Cell Biology Trading, Hamburg, Germany). Proteins were injected into the cytoplasm at 4 μg/μl in the case of WASp polyproline and dynamin II polyproline domain, at 2 μg/μl in the case of the CIP4 deletion mutants, and at 3 μg/μl in the case of WASp VC domain. Control injections were performed with GST at similar concentrations. Injected cells were identified by labeling coinjected rat IgG (5 mg/ml; Dianova) with FITC labeled goat anti-rat IgG antibody (Dianova) or by coinjecting FITC-labeled rat IgG (10 mg/ml, Dianova).

RESULTS

The actin and microtubule cytoskeletons of primary human macrophages show a close spatial relationship

To examine the relationship between podosomes and microtubules in primary human macrophages, we first performed a series of (immuno-)fluorescence stainings (Fig. 1). Mature quiescent macrophages (5 to 7 days old) show a radial symmetry and numerous f-actin-rich dots corresponding to podosomal adhesion structures which surround a small podosome-free area in the center (Fig. 1A; Linder et al., 1999). Microtubules extend from the perinuclearly located microtubule organizing center into the peripheral parts of the cell (Fig. 1B). Serial horizontal sections revealed that microtubules originate apically and then project into the basal plane of the cell which contains the podosomes (Fig. 1C,F). In some cases, microtubules seem to directly end at or to contact podosomes (Fig. 1C,F). It is worthy of notice that both, central parts of cells which are normally podosome-free (Fig. 1A) and also randomely occuring podosome-free areas (not shown), are devoid of microtubule ends (Fig. 1C). Considering the large volume of the dome-shaped central part of a macrophage, it is remarkable that both, podosomes and microtubule ends, are found predominantly in the ventral plane of the cell. All these observations point to a close spatial relationship between podosomes and microtubules.

Microtubules are necessary for podosome formation during monocyte adhesion

We next asked whether the close association of podosomes and microtubules might reflect a functional relationship. At first, we were interested in the potential role of microtubules in initial podosome formation during early adhesion of monocytes. To address this question, freshly isolated monocytes were seeded onto glass coverslips and allowed to adhere. Podosome formation, as evidenced by dot-like f-actin staining, started to be detectable after 2 hours (Fig. 2A). At this time point, adhering monocytes showed a well-developed microtubule system (Fig. 2B). When an aliquot of monocytes was seeded in medium containing the microtubule-depolymerising drug nocodazole, adherence to glass coverslips was retained and, as expected, these cells contained no microtubules (Fig. 2D). Additionally, they also failed to develop podosomes, even after 6 hours of incubation (Fig. 2C). After placing the nocodazole-treated monocytes in drug-free medium for 2 hours, the cells regained their microtubule system and also developed podosomes (Fig. 2E,F).

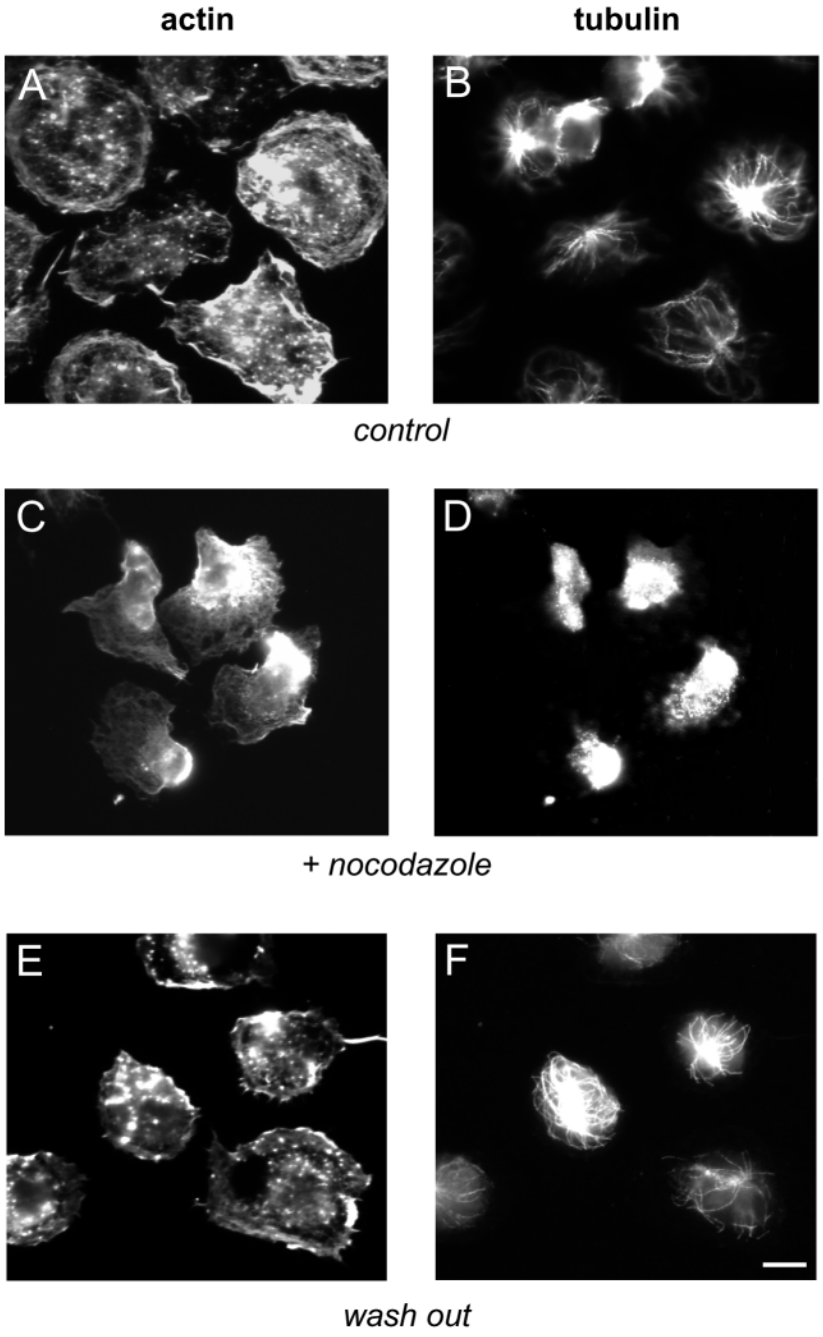

Fig. 2. Intact microtubules are necessary for podosome formation during monocyte adhesion. Fluorescence micrographs of freshly isolated primary human monocytes seeded onto glass coverslips. F-actin stained with rhodamine phalloidin (A,C,E), tubulin stained with anti-β-tubulin antibody (B,D,F). Control cells were seeded in standard medium and fixed 2 hours after seeding (A,B). At this timepoint, f-actin rich podosomes were readily visible. An aliquot of cells was seeded in medium containing nocodazole (1 μmol) and fixed 2 hours after seeding (C,D). Nocodazole-induced depolymerisation of microtubules (D) was accompanied by an inability of cells to assemble podosomes (C). An additional aliquot of cells was seeded in medium containing nocodazole (1 μmol) and after 2 hours placed in drug-free medium for an additional 2 hours (E,F). Reformation of microtubules (F) was accompanied by podosome assembly (E). Bar, 10 μm.

We also performed experiments with the microtubule-depolymerizing agents benomyl and vinblastine. Especially vinblastine uses a different mode of microtubule-depolymerization compared to nocodazole by reorganizing tubulin into paracrystals (Starling, 1976). Monocytes seeded in medium containing benomyl or vinblastine behaved indistinguishably from those treated with nocodazole (not shown).

We therefore conclude that the inability of monocytes to develop podosomes in these experiments is a direct result of the depolymerization of their microtubule cytoskeleton.

Podosome reformation in adherent macrophages is dependent on intact microtubules

Having shown that podosome formation in monocytes undergoing adhesion depends on microtubules, we asked whether this also holds true for the reformation of podosomes in mature adherent macrophages. Podosomes in macrophages are highly dynamic organelles undergoing constant disassembly and reformation with a half-life of 2-12 minutes (Kanehisa et al., 1990). This process clearly shows higher dynamics than podosome formation during monocyte adhesion and might therefore also have other characteristics.

To study podosome reformation in mature macrophages, we first disassembled podosomes by two different means: (1) by disruption of the f-actin rich core using the f-actin-depolymerizing drug cytochalasin D (2 μM for 1 hour) or (2) by blocking tyrosine phosphorylation using tyrosine kinase inhibitor PP2 (25 μM for 1 hour; S. Linder et al., unpublished observation). Both treatments resulted in an almost complete disappearance of podosomes (cells containing podosomes: 9.3±4.1% of cytochalasin D-treated, and 10.0±3.7% of PP2-treated cells vs 94±4.0% in controls; Fig. 3A,G,M), but left the microtubule system unaffected (Fig. 3D,J). Podosome disruption was often associated with prominent actin clumping. Thereafter, cells were allowed to recover for 1.5 hours in drug-free medium, which led to reformation of podosomes (65.3±6.4% of cytochalasin D-treated, and 74.0±9.3% of PP2-treated cells showed podosomes; Fig. 3B,H,M). Longer recovery periods (>1.5 hours) resulted in podosome reformation in almost all cells.

Next we added nocodazole during the 1.5 hours recovery period to investigate the influence of microtubules on podosome reformation. In the presence of nocodazole, microtubules were disrupted (Fig. 3F,L) and cells failed to recover their podosomes (cells containing podosomes: 18.0±6.2% of cytochalasin D-treated and 18.7±1.6% of PP2-treated cells; Fig. 3C,I,M). Cells treated with vinblastine during the recovery period produced similar results (not shown).

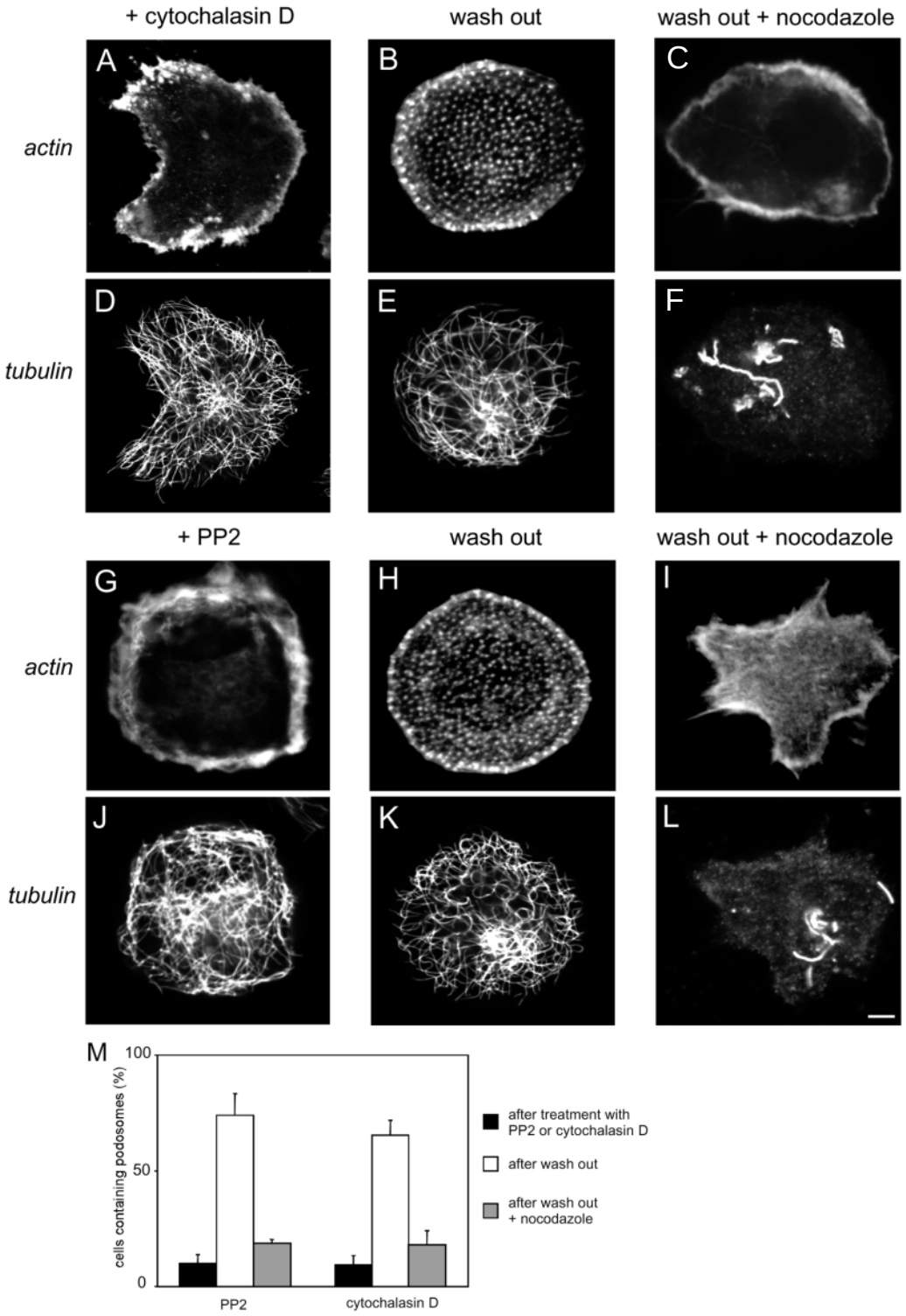

Fig. 3. Podosome reformation in adherent macrophages is dependent on intact microtubules. Fluorescence micrographs of primary human macrophages, cultured for 6 days on glass coverslips, f-actin stained with rhodamine phalloidin (A-C; G-I), tubulin stained with anti-β-tubulin antibody (D-F; J-L). Podosomes were disrupted by treatment for 1 hour with f-actin-depolymerizing cytochalasin D (A-F) or tyrosine kinase inhibitor PP2 (G-L). Cells were either directly fixed after incubation (A,D,G,J), placed in drug-free medium for an additional 1.5 hours, either without further additives (B,E,H,K) or containing 1 μmol nocodazole (C,F,I,L). Cells in drug-free medium and intact microtubules readily formed podosomes (B,E,H,K), while reformation of podosomes was inhibited by nocodazole-induced disassembly of microtubules (C,F,I,L). Bar, 10 μm (A-L). (M) Diagram showing percentage of cells containing podosomes after treatment with cytochalasin D or PP2 (black bars), after wash out of these drugs (white bars), and after wash out with nocodazole added (grey bars). For each value, 50 cells of 3 independent experiment were counted. Values for untreated cells without podosomes were below 3%.

Staining for vinculin showed that reappearance of the actin-rich podosome core in the washout experiments is also accompanied by the reformation of the vinculin-containing outer ring of individual podosomes (Fig. 4A-C). Dash-like vinculin staining in the cell periphery indicating focal adhesions, was minimal and not observed in most cases. In contrast, upon addition of nocodazole, vinculin is recruited into large and numerous focal adhesions (Fig. 4D-F), as described for other cell types (Bershadsky et al., 1996; Enomoto, 1996).

These findings demonstrate that the functional association with microtubules is fundamentally different between podosomes and focal adhesions. To further investigate the similarities and differences between podosomes and focal adhesions, we performed the podosome-reformation experiments described above also in the presence of RGDS peptide (Fig. 5). This tetrapeptide constitutes the integrin-binding region in the extracellular matrix protein fibronectin and can inhibit the formation of focal adhesions in NRK cells

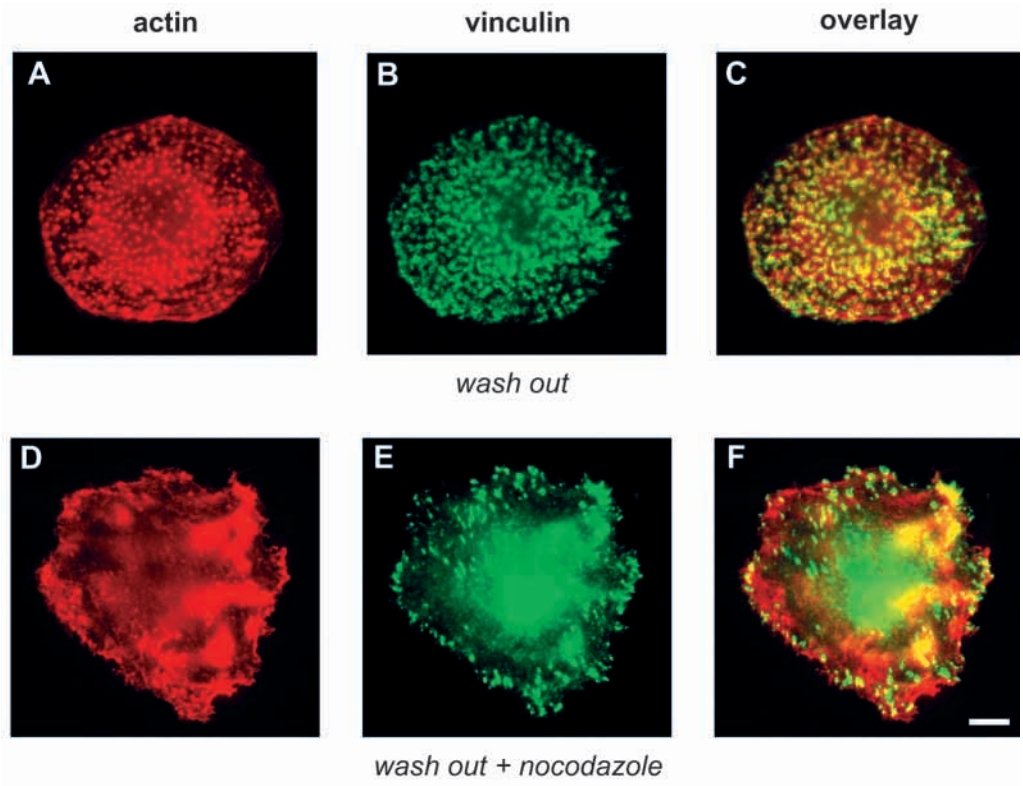

Fig. 4. Podosomes and focal adhesions differ in their dependence on microtubules. Fluorescence micrographs of primary human macrophages, cultured for 6 days on glass coverslips, f-actin stained with rhodamine phalloidin (A,D), red color, vinculin stained with anti-vinculin antibody (B,E), green color, overlay of A and B (C), overlay of D and E (F). Podosomes of primary human macrophages were disrupted by treatment with cytochalasin D. The drug was washed out either without further additives (A-C) or in the presence of nocodazole (D-F). Podosomes reform in the presence (A-C) but not in the absence of microtubules (D-F). In contrast, microtubule depolymerization by nocodazole leads to formation of large and numerous focal adhesions (D-F). Bar, 10 μm.

(Pierschbacher and Ruoslahti, 1984). Consistently, in macrophages RGDS (1 mM) was able to inhibit focal adhesions that formed when nocodazole was present in the podosome reformation assay described above (Fig. 5A-D). In contrast, reformation of podosomes (in the absence of nocodazole) proceeded normally in the presence of RGDS (1 mM; Fig. 5E-H), suggesting greatly different susceptibilities of podosomes vs focal adhesions to fibronectin-derived peptides. In sum, we conclude that the reformation of podosomes in macrophages is dependent on an intact microtubule cytoskeleton. The pro-formatory role of microtubules for podosomes is in clear contrast to their anti-formative effect upon focal adhesions. Additionally, podosome reformation in mature adherent macrophages is not influenced by addition of 1 mM RGDS peptide, which, in contrast, readily suppresses the formation of focal adhesions.

Reformation of podosomes can be blocked by microinjection of the WASp polyproline domain

Looking for a mechanism to explain the microtubule-dependence of podosome formation, we considered two previous observations: (1) WASp controls podosome formation (Linder et al., 1999), and (2) WASp was found to bind to microtubules via the CDC42 interacting protein 4 (CIP4; Tian et al., 2000). Indeed, under certain staining conditions, WASp can be shown to localize to microtubules in primary human macrophages (Fig. 6). Interestingly, the WASp-CIP4 interaction has been proposed to be involved in the assembly of adhesive structures. Since binding of CIP4 to WASp is mediated by the WASp polyproline domain, we created a GST-fusion product of the WASp polyproline domain (Fig. 7A) and microinjected it into primary macrophages.

Because the WASp effector and actin nucleator Arp2/3 complex can be purified on polyproline columns (Machesky et al., 1994), we first tested for interaction of the WASp polyproline domain with the Arp2/3 complex in GST-pull down assays of macrophage lysates. As a control, we used a GST fusion with the WASp acidic domain which precipitates the Arp2/3 complex (Fig. 7B; Linder et al., 2000). The WASp polyproline domain, however, showed no interaction with the Arp2/3 complex (Fig. 7B).

When microinjection into primary macrophages is performed with high enough pressure, most of the injected cells transiently lose their podosomes, as judged by staining for actin, WASp and vinculin. Subsequent recovery of podosomes is normally accomplished within 30-60 minutes (S. Linder et al., unpublished observation). We took advantage of this microinjection artefact to study the reformation of podosomes. The majority of macrophages injected with GST lost their podosomes within 2 minutes after microinjection (cells containing podosomes: 24.3±7.5%; Fig. 8A,G). Within 1 hour, most of the microinjected macrophages reformed their podosomes (91.7±2.3%; Fig. 8B,G). Macrophages injected with WASp polyproline domain also lost their podosomes (cells containing podosomes: 29.7±13.5%; Fig. 8D,G), however, within the following hour, no significant podosome reformation occured (35.0±10.2%; Fig. 8E,G). This not only holds true for the podosomal localization of actin, but also for that of WASp and vinculin (not shown). Thus, the WASp polyproline domain completely prevented reassembly of podosomes. This effect was not due to damage of the microtubule system, since both in GST- and WASp polyproline domain-injected cells, the tubulin cytoskeleton seemed unaffected (Fig. 8C,F). Values for macrophages microinjected with the polyproline domain of dynamin II used as a control were similar to injections with GST for initial loss of

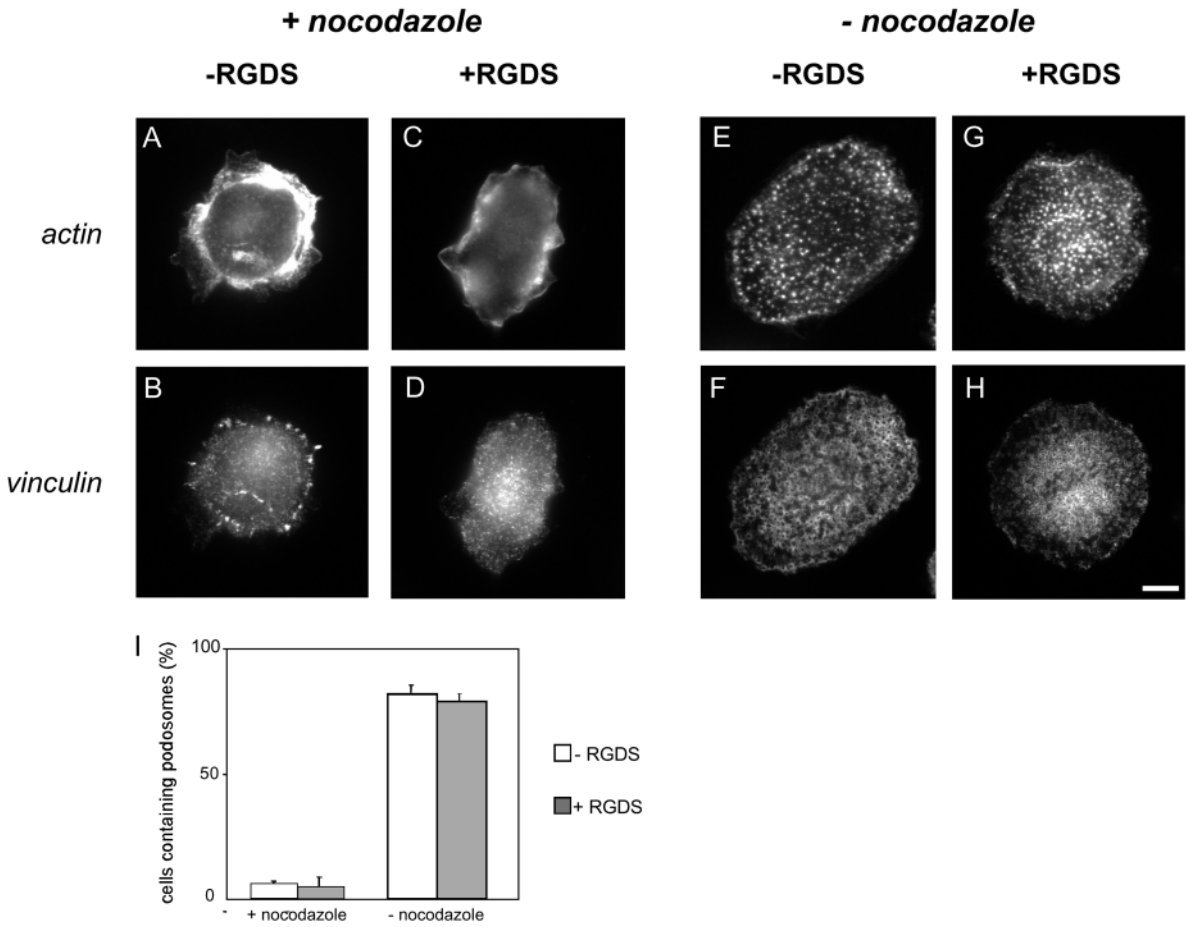

Fig. 5. Influence of microtubules and RGDS peptides on podosomes vs. focal adhesions. Immunofluorescence micrographs of 6-day-old primary human macrophages, f-actin stained with rhodamine-phalloidin (A,C,E,G), vinculin stained with anti-vinculin antibody (B,D,F,H). Podosomes of primary human macrophages were disrupted by treatment with cytochalasin D. After 1 hour, cells were washed with (A-D) or without (E-H) addition of nocodazole, and with (C,D,G,H) or without (A,B,E,F) addition of 1 mM RGDS peptide, respectively. Bar, 10 μm, for all panels. (I) Diagram showing percentage of cells containing podosomes after the respective treatments. White bars: without RGDS peptide; grey bars: RGDS peptide added. For each value, 30 cells of 3 independent experiments were evaluated. Values for untreated cells without podosomes were below 3%.

podosomes (cells containing podosomes: 18.7±2.3%; Fig. 8G) and podosome reassembly (86.0±6.6%; Fig. 8G). Interestingly, dynamin II has recently been shown to be an integral part of podosomes in Rous sarcoma virus-transformed BHK21 cells and in osteoclasts (Ochoa et al., 2000).

We conclude that macrophages microinjected with WASp polyproline domain fail to reassemble podosomes. This effect seems to be specific for the polyproline domain of WASp and appears to be due neither to damage of the microtubule system nor to Arp2/3 sequestration.

Reformation of podosomes can be blocked by microinjection of CIP4 deletion constructs

We went on to look for a possible involvement of CIP4 in podosome formation. To this purpose, we microinjected primary macrophages with deletion mutants of CIP4, which either lack the WASp-binding domain (CIP4 (1-481); Fig. 9) or the microtubule-binding domain (CIP4 (118-545; Fig. 9). Most control cells injected with GST lost their podosomes (cells containing podosomes: 20.0±8.7%; not shown), but recovered them within 1 hour (cells containing podosomes: 93.3±2.7%; not shown). Cells injected with the CIP4 (1-481) deletion construct also mostly lost podosomes (cells containing podosomes: 19.0±5.7%; Fig. 9B). However, in clear contrast to the GST controls, they failed to recover them within 1 hour (cells containing podosomes: 15.7±8.3%; Fig. 9C). Microinjections of the CIP4 (118-481) construct showed similar values for initial loss of podosomes (cells containing podosomes: 30.0±9.3%; Fig. 9D). After 1 hour, the pool of injected cells not only failed to recover podosomes but showed a further loss of these structures (cells containing podosomes: 3.0±2.7%; Fig. 9E).

Taken together, macrophages microinjected with CIP4 deletion constructs either deficient in the WASp-binding or in the microtubule-binding domain fail to reassemble podosomes.

Correlated defects in the actin and tubulin cytoskeletons of macrophages

Podosome formation in monocytes and macrophages shows a dependency on the microtubule system and is abrogated in the absence of intact microtubules. In turn, we found that the absence of podosomes in WAS macrophages often coincides

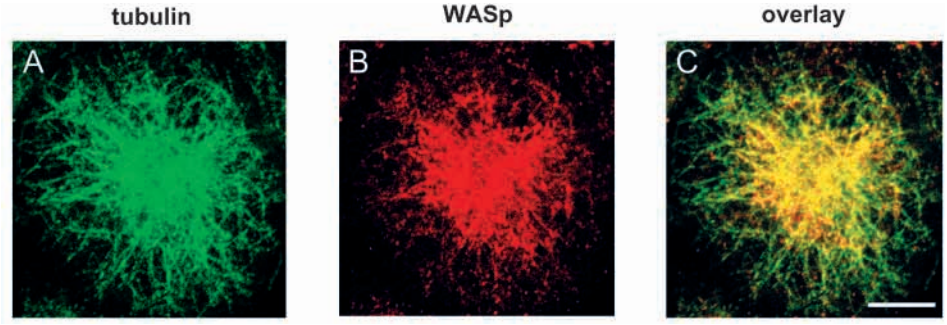

Fig. 6. WASp can localize at microtubules in primary human macrophages. (A-C) Confocal laser scanning micrographs showing apical part of a 7-day-old primary human macrophage, gained by superimposition of each time 4 horizontal sections. Tubulin stained with anti-α-tubulin antibody, green color (A), WASp stained with anti-WASp antibody, red color (B), overlay of A and B (C), yellow color indicates colocalization. Bar, 10 μm.

with a disturbed microtubule cytoskeleton, both in its overall shape and also on the level of orientation of individual microtubules (Fig. 10A-C). Irregularities in the overall shape are clearly due to the more bizarre shapes of WAS macrophages. Internal disturbance of micortubule networks, however, seems to be associated with the formation of irregular F-actin clusters (Fig. 10C, arrows).

We therefore wondered whether the absence of podosomes and/or formation of actin clusters could be responsible for the abnormalities observed in the microtubule system of these WAS macrophages. A first hint comes from the fact that WASp polyproline-injected macrophages fail to reform podosomes, but microtubules seem unaffected (Fig. 8F). To investigate another condition where podosome absence and actin clusters are present at the same time, we microinjected the WASp VC domain into healthy macrophages. The VC domain always disrupts podosomes and in about 25%-75% of injected cells (depending on incubation time and age of culture) also leads to the formation of actin clumps (Linder et al., 1999) that resemble the irregular actin clusters observed in a subpopulation of WAS macrophages. VC domain-injected macrophages with disrupted podosomes but no actin clusters showed an undisturbed microtubule system (Fig. 10D-F). However, macrophages containing VC domain-induced actin clumps displayed dramatic rearrangements of the tubulin cytoskeleton. In these cells, microtubules were mostly arranged around actin clumps (Fig. 10G-H). Therefore, not podosome disruption but aberrant actin polymerization resulting in irregular actin clusters seems to force microtubules to reorient themselves. This circumstance most likely is also the cause when the internal organisation of microtubule systems is disturbed in macrophages from WAS patients.

DISCUSSION

In this study, we show that the actin-rich podosomal adhesions and the microtubule system of primary human macrophages share a close spatial and functional relationship. Microtubules mostly end in the cell periphery where podosomes are located, and in some cases, they seem to directly contact each other. An earlier study in osteoclasts showed that partial disassembly of microtubules leads to a more random pattern of podosome distribution (Babb et al., 1997). All of these findings suggest that there exists a functional relationship between podosomes and microtubules.

Indeed, we find that in primary human monocytes and macrophages the formation of podosomes depends on an intact microtubule system. Freshly isolated monocytes undergoing adhesion fail to develop podosomes when treated with microtubule-depolymerizing drugs. After wash-out of the respective drug and reformation of the microtubule system, podosomes reform accordingly. We gained similar results with mature adherent macrophages where podosomes constantly disassemble and reform. Therefore, an intact microtubule cytoskeleton is necessary for both, the initial formation of podosomes during monocyte adhesion and also for podosome reformation in mature adherent macrophages.

Microtubule depolymerization or shortening has been shown to activate the small GTPase Rho, which ultimately results in

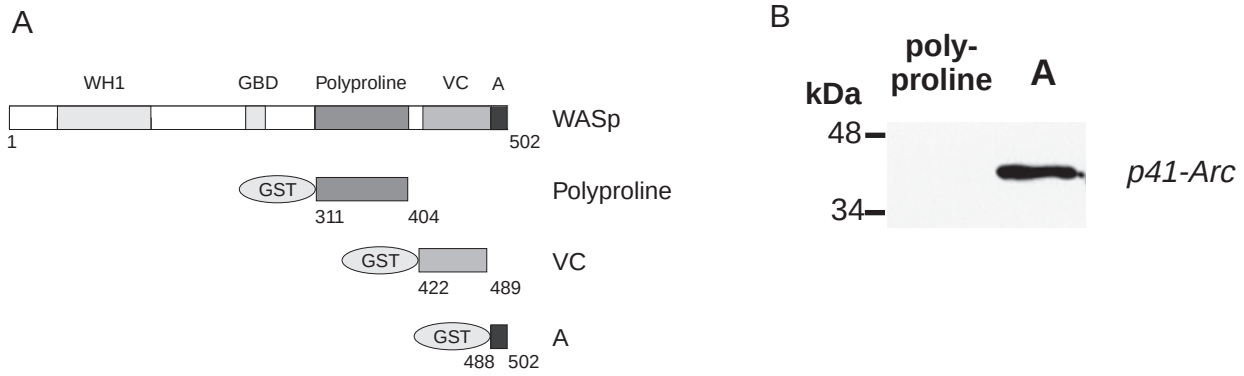

Fig. 7. (A) Domain organisation of WASp: WASp homology domain 1 (WH1), GTPase-binding domain (GBD), polyproline domain, C-terminal domain (containing verprolin-like and central C-terminal ('VC') and acidic ('A') domains). Numbers indicate first and last amino acid of WASp domains as part of the GST fusion proteins used in this study. (B) The WASp polyproline domain does not bind the Arp2/3 complex. Western blot of GST pull-down assays performed with WASp polyproline domain ('polyproline') and WASp acidic domain ('A'), developed with polyclonal antibody against p41-Arc subunit of the Arp2/3 complex. Molecular mass in kDa is indicated on the left.

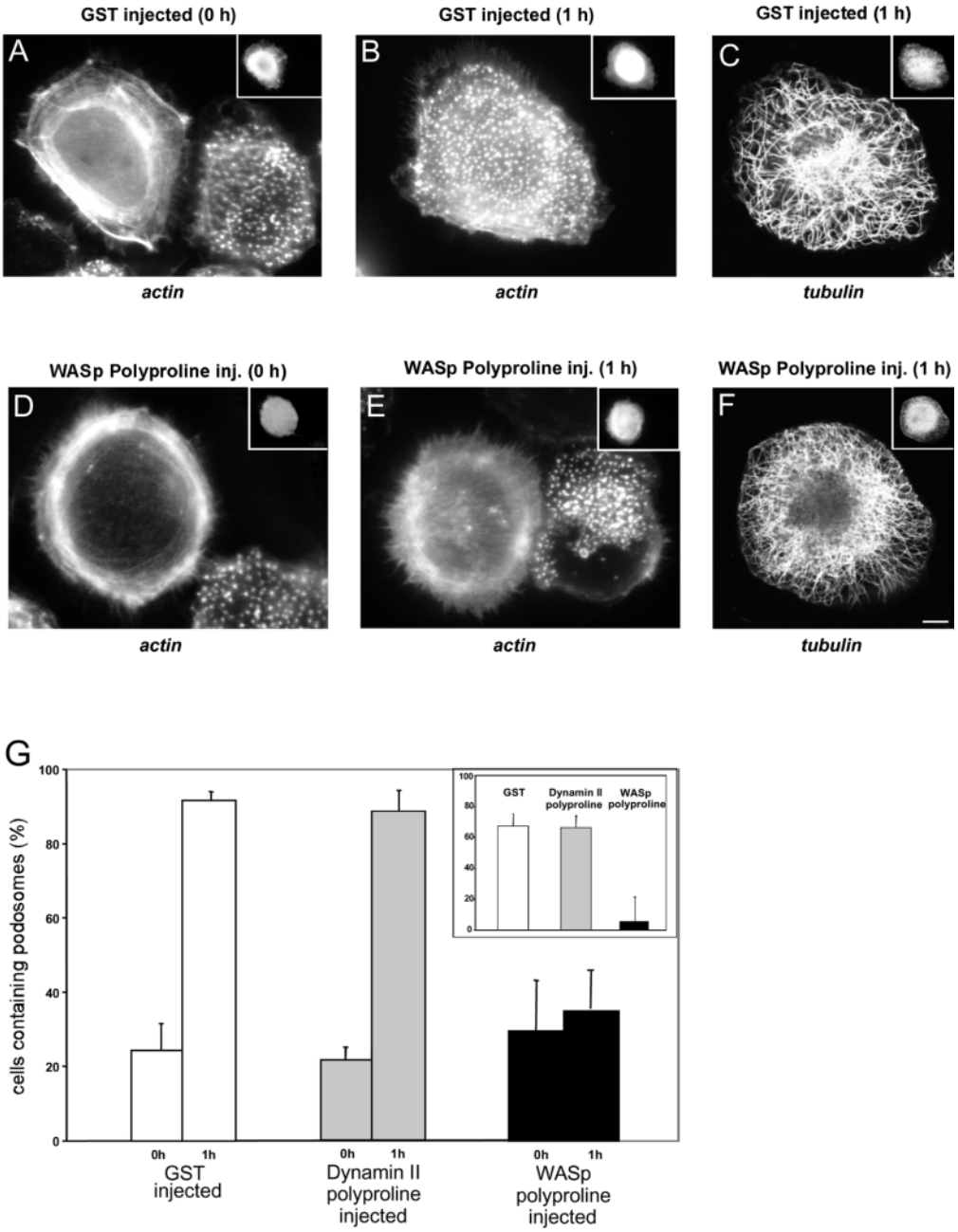

Fig. 8. Reformation of podosomes can be blocked by microinjection of the WASp polyproline domain. Fluorescence micrographs (A,B,D,E) and laser scanning confocal micrographs (C,F) of microinjected human macrophages, cultured for 6 days on glass coverslips (A-C), f-actin stained with rhodamine phalloidin (A,B,D,E), tubulin stained with anti-α-tubulin (C,F), insets showing rat igG staining used as injection marker. Macrophages were microinjected with GST as a control (A-C) or with a GST-fusion protein of WASp polyproline domain (D-F). Cells were fixed either directly after microinjection (A,D) or after an additional 1 hour of incubation (B,C,E,F). Microinjection of macrophages with high enough pressure mostly results in loss of podosomes (A,D). GST-injected cells mostly recover podosomes 1 hour post-injection (B), while WASp polyproline-injected cells mostly fail to do so (E). This effect is not due to damage of the microtubule system, as microtubules in injected cells appear unaffected (C,F). (Note: apparent differences in cell shape of actin and anti-rat igG staining in (D) are due to contorted cellular morphology). Bar, 10 μm. (G) Diagram showing percentage of cells containing podosomes 0 hour or 1 hour after injection with GST (white bars), with dynamin II polyproline domain (grey bars) or with WASp polyproline domain (black bars). Inset showing percentages of injected cells that recover podosomes. For each value, 30 cells of 3 indepedent experiments were counted.

increased cell contractility (Kolodney and Elson, 1995; Chrzanowska-Wodnicka and Burridge, 1996; reviewed by Waterman-Storer and Salmon, 1999). Primary human monocytes and macrophages show a similar phenomenon, as treatment with microtubule-depolymerizers is accompanied by a reduction of their adhesive surface probably due to cell contraction/reduced spreading. In many cell types, microtubule depolymerization is also accompanied by the formation and growth of focal adhesions (Lloyd et al., 1977; Kaverina et al., 1998), and we found that this holds true also for macrophages. The fact that microtubule depolymerization does not lead to podosome formation but, on the opposite, inhibits podosome formation, highlights the difference between podosomes and focal adhesions. These structures differ further in their susceptibility to fibronectin-derived peptides. However, podosomes have been shown to contain integrin clusters, among them also β1 chains that are part of the fibronectin receptor (Marchisio et al., 1988). We found that the presence of 1 mM RGDS slightly delays initial formation of podosomes in monocytes (S. Linder et al., unpublished). Integrins and integrin binding to extracellular matrix therefore undoubtedly play a role in podosome architecture and formation, but one clearly different from that in focal adhesions.

To explain the dependency of podosomes upon microtubules in more molecular terms, we combined two observations: (1) WASp is a key regulator of podosomes and its absence in WAS macrophages results in a failure in podosome assembly (Linder et al., 1999) and (2) WASp can bind via its polyproline domain to CIP4, which, in turn, binds to microtubules (Tian et al., 2000). In a series of microinjection experiments we were able to show that reformation of podosomes could be blocked by injection of a GST-WASp polyproline fusion protein. This

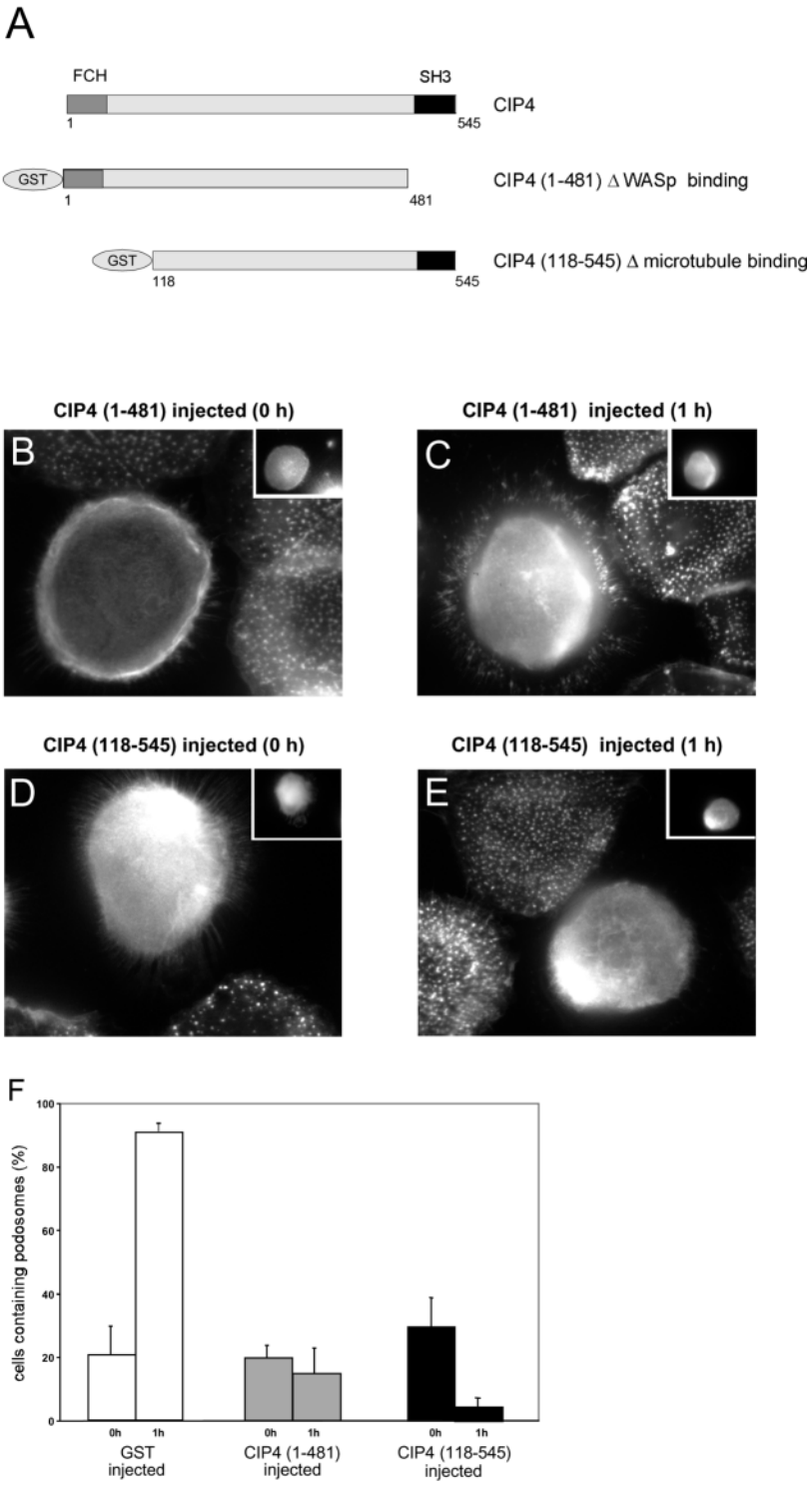

Fig. 9. Reformation of podosomes can be blocked by microinjection of CIP4 deletion mutants. (A) Domain organisation of CIP4: microtubule-binding Fer and Fes/Fps homology domain (FCH), WASp-binding Src homology 3 domain (SH3). Numbers indicate first and last amino acid of CIP4 domains as part of the GST fusion proteins used in this study. (B-E) (Immuno-) fluorescence micrographs of 7-day-old primary human macrophages, f-actin stained with rhodamine-phalloidin, insets showing rat igG staining used as injection marker. Macrophages were microinjected with GST-fusion proteins of CIP4 either lacking the WASp-binding SH3 domain (CIP4 (1-481); B,C) or the microtubule-binding FCH domain (CIP4 (118-545); D,E). Cells were fixed either directly after microinjection (B,C) or after an additional 1 hour of incubation (D,E). Microinjection of macrophages with high enough pressure mostly results in loss of podosomes (B,D), cells injected with either CIP4 deletion mutant mostly fail to recover podosomes (C,E). Bar, 10 μm. (F) Diagram showing percentage of cells containing podosomes 0 hours or 1 hour after injection with GST (white bars), with CIP4 (1-481) (grey bars) or with CIP4 (118-545) (black bars). For each value, 30 cells of 3 indepedent experiments were counted.

effect is not due to damage of the microtubule system and it also appears to be specific for the polyproline domain of WASp.

Beside CIP4, the WASp polyproline domain has been shown to bind to a variety of proteins, among them tyrosine kinases of the Src-family (Banin et al., 1996; Zhu et al., 1997) and the adaptor proteins Grb2 and Nck (Rivero et al., 1995; Cory et al., 1996). Ultimately, we cannot exclude the possibility that microinjection of the WASp polyproline domain has effects on these molecules which could lead to the observed failure in podosome formation. However, because many WASp family binding partners like Grb2 (Carlier et al., 2000) have been shown to have modulatory but not pivotal roles in the regulation of f-actin assembly, we propose that correct positioning of WASp via microtubules could be a key event in podosome formation.

This model gains further credibility by the fact that also microinjected GST-fusion proteins of CIP4 deleted either in the WASp-binding or the microtubule-binding domain induce a failure in podosome reassembly, similar to the effect of the WASp polyproline domain. In the case of the CIP4 mutant lacking the microtubule-binding domain, even cells which did not lose their podosomes immediately upon microinjection, lost these upon further incubation.

CIP4 has been identified as a binding partner of active CDC42 (Aspenström, 1997), but CDC42 binding is not necessary for CIP4 to mediate localization of WASp to microtubules (Tian et al., 2000). Therefore, a hypothetical binding of WASp to and transport along microtubules in vivo would not necessarily involve altering of its activation status. In a possible scenario, WASp could be transported in its inactive state and, only upon arrival at its podosomal destination, be activated to induce Arp2/3-dependent actin polymerization.

Having shown the important effect of microtubules on podosomes, we went on to look for a possible influence of podosomes on microtubules. Indeed, in WAS macrophages where podosomes are absent, microtubule systems often seem to be misformed. This effect may be partly due to the restrictions imposed on microtubules by the often rather bizarre

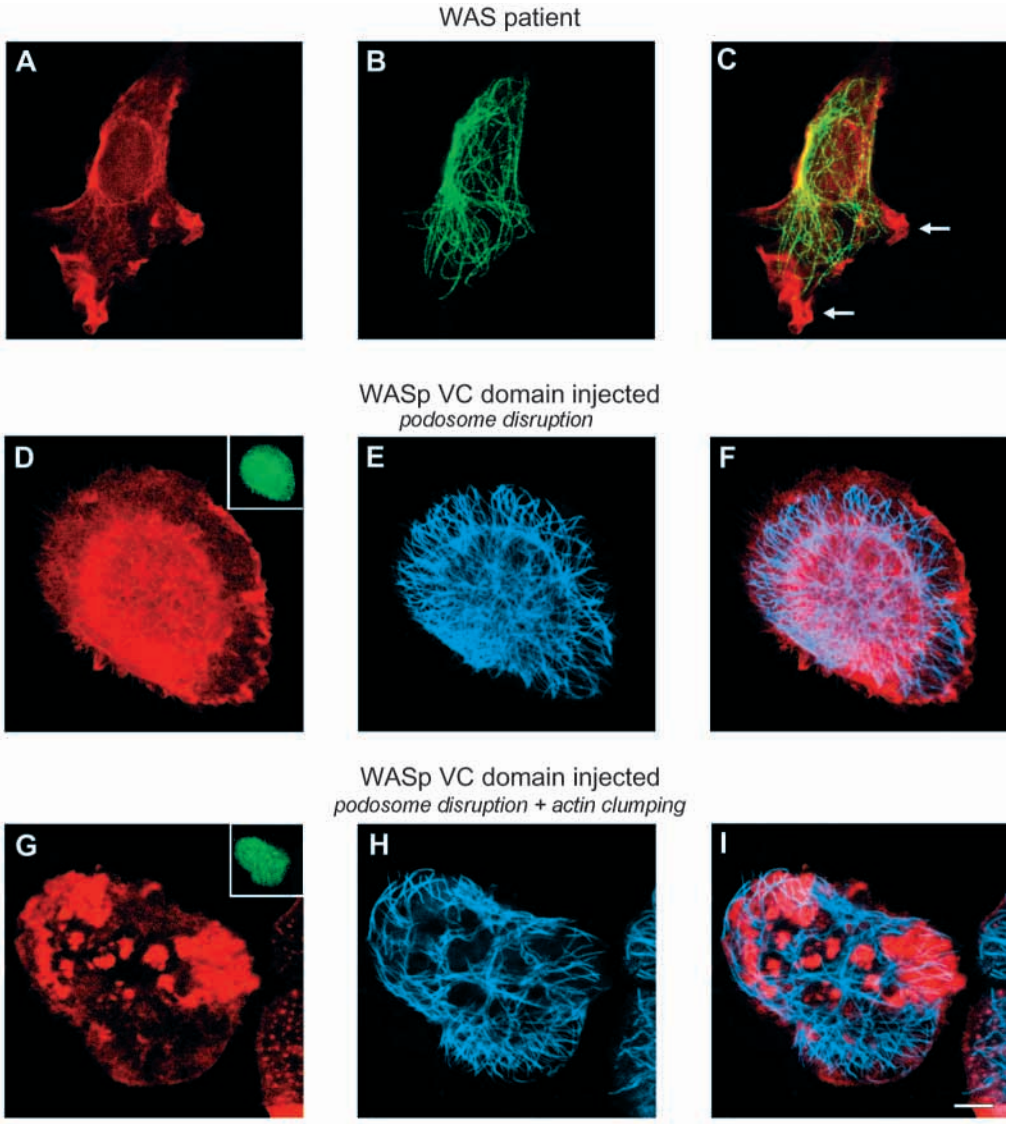

Fig. 10. Disturbance of microtubules in WAS macrophages or WASp VC domain-injected macrophages is due to spatial constraints of an aberrant actin cytoskeleton and not a direct effect of podosome absence. Confocal laser scanning micrographs of ventral parts of cells, f-actin stained with rhodamine-phalloidin, red color (A,D,G), tubulin stained with anti-α-tubulin antibody, blue color (B,E,H), insets showing rat igG staining as microinjection marker, green color (D,G), overlay of A and B (C), of D and E (F), and of G and H (I). (A-C) Macrophage from WAS patient, typically devoid of podosomes, cultivated for 6 days on a glass coverslip. WAS macrophage shows abnormal development of microtubule system, associated with aberrant actin polymerization (arrows). (D-I) Macrophages from healthy donor, cultivated for 6 days on glass coverslips, injected with WASp VC domain, leading to podosome disruption (D-F), which can be accompanied by actin clumping (G-I). Microinjection with WASp VC domain with subsequent podosome disruption does not necessarily lead to disturbance of microtubule cytoskeleton (D-F). Only the secondary effect of actin clumping forces microtubules to reorient themselves (G-I). Bar, 10 μm.

shapes of WAS macrophages. However, in many cases not only the overall shape but also the intracellular distribution of the microtubule network is disturbed. To test whether the latter is due to absence of podosomes or to sterical hindrance by actin clusters, we microinjected macrophages from healthy donors with the WASp VC-domain wich induces both, podosome disruption and also actin clumping (Linder et al., 1999). We could show that in macrophages where actin clumps had formed, microtubules were forced to reorient themselves into unusual patterns. On the other hand, macrophages without podosomes but containing no actin clumps showed normally developed microtubule systems. Therefore, aberrancies in the microtubule system are not a direct result of the absence of podosomes, but are due to sterical hindrance imposed through associated actin clumping, both in VC-domain injected macrophages and probably also in WAS macrophages.

In sum, our study provides a further example of the complex interplay between microtubules and actin-based structures, here podosomes in primary macrophages. It also stresses the fact that podosomes are fundamentally different from other adhesion structures like focal adhesions. In particular, we show that (1) podosome formation in primary human monocytes and macrophages depends on microtubules but not vice versa and (2) microinjection of the WASp polyproline domain and of CIP4 deletion mutants results in a failure of podosome assembly.

Release of actin-regulatory factors from microtubules, for example the small GTPase Rac (Best et al., 1996) or the Rho/Rac guanosine nucleotide exchange factor Vav (Huby et al., 1995; Fernandez et al., 1999), has been proposed earlier. Given the dependency of podosomes on microtubules, deposition of a microtubule-bound factor at sites of podosome formation would be in line with these thoughts. The possible involvement of WASp in microtubule-dependent podosome assembly suggests that further research on the potential role of WASp as such a factor should be of merit.

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