N-WASP deficiency impairs EGF internalization and actin assembly at clathrin-coated pits

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Summary
WASP and WAVE family proteins promote actin polymerization by stimulating Arp2/3-complex-dependent filament nucleation. Unlike WAVE proteins, which are known to drive the formation of protrusions such as lamellipodia and membrane ruffles, vertebrate cell functions of WASP or N-WASP are less well established. Recent work demonstrated that clathrin-coated pit invagination can coincide with assembly of actin filaments and with accumulation of N-WASP and Arp2/3 complex, but the relevance of their recruitment has remained poorly defined. We employed two-colour total internal reflection microscopy to study the recruitment and dynamics of various components of the actin polymerization machinery and the epidermal growth factor receptor signalling machinery during clathrin-coated pit internalization in control cells and cells genetically deficient for functional N-WASP. We found that clathrin-coated pit endocytosis coincides with the recruitment of N-WASP, Arp2/3 complex and associated proteins, but not of WAVE family members. Actin accumulation at clathrin-coated pits requires the Arp2/3 complex, since Arp2/3 complex sequestration in the cytosol abolished any detectable actin assembly. The absence of N-WASP caused a significant reduction in the frequencies of actin and Arp2/3 complex accumulations at sites of clathrin-coated pit invagination and vesicle departure. Although N-WASP was not essential for Arp2/3-complex-mediated actin assembly at these sites or for EGF receptor-mediated endocytosis, N-WASP deficiency caused a marked reduction of EGF internalization.

We conclude that the assembly of WASP subfamily proteins and associated factors at sites of clathrin-coated pit invagination amplifies actin accumulations at these sites promoting efficient internalization of ligands via clathrin-mediated endocytosis.

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Key words: N-WASP, Arp2/3 complex, Actin, EGF receptor, Clathrin, Endocytosis

Introduction
Reorganisation of the actin cytoskeleton is essential for a variety of fundamental metazoan processes, including the formation of protrusions during migration, establishment and maintenance of cell-substrate or cell-cell adhesion and cytokinesis (Mitchison and Cramer, 1996; Small et al., 2002; Wang, 2001; Wehrle-Haller and Imhof, 2002). More recently, the actin cytoskeleton has also been implicated in a number of vesicle trafficking processes and has appeared to drive the motility of certain particles such as the intracellular pathogens Listeria and Shigella and of endosomal and lysosomal vesicles (Frischknecht and Way, 2001; Taunton, 2001). In budding yeast, actin reorganisation is essential for endocytosis, mediated most probably by cortical actin patches (Engqvist-Goldstein and Drubin, 2003; Kaksonen et al., 2003; Munn, 2001). In animal cells, the relevance of actin assembly and turnover for endocytic processes is less firmly established (reviewed by Engqvist-Goldstein and Drubin, 2003; Merrifield, 2004), although recent experiments indicate a role at different stages of clathrin pit formation and internalization (Yarar et al., 2005).

Actin polymerization is initiated by the nucleation of actin filaments, which is catalysed, for instance, by the actin related protein 2/3 complex (Arp2/3 complex) mediating the amplification and branching of actin filaments at sites of dynamic actin reorganization (Welch and Mullins, 2002). Arp2/3-complex-dependent actin filament assembly is presumed to be essential for the majority of dynamic actin rearrangements occurring at the plasma membrane such as the internalization step of endocytosis in budding yeast (reviewed by D’Hondt et al., 2000; Engqvist-Goldstein and Drubin, 2003; Munn, 2001; Shaw et al., 2001). For lamellipodium protrusion and membrane ruffling (Small et al., 2002; Welch and Mullins, 2002) or for podosome formation in transformed fibroblasts and in cells of the haematopoietic lineage (Thrasher, 2002). The intrinsic actin nucleation activity of the Arp2/3 complex is comparatively low, but is drastically increased upon interaction with so-called nucleation promoting factors, the...
most prominent of which are grouped in the WASP and WAVE family proteins (Stradal et al., 2004; Vartiainen and Machesky, 2004; Welch and Mullins, 2002). In mammals, this protein family comprises the haematopoietic WASP, the ubiquitously expressed so called neural WASP (N-WASP) and three WAVE isoforms. A large body of in vitro biochemical and structural biological investigations have revealed the complexity of regulation of WASP subfamily proteins (Ho et al., 2004; Stradal et al., 2004; Takenawa and Miki, 2001). For instance, both N-WASP and WASP can directly interact with small GTPases of the Rho family such as Cdc42 and with the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2/PtdIns(4,5)P2), which in synergy are capable of releasing the autoinhibitory conformation of these proteins (Kim et al., 2000; Prehoda et al., 2000). However, WASP subfamily proteins are regulated, in addition, by a multitude of additional proteins, prominent examples of which include members of the WASP interacting protein (WIP) family (Aspenstrom, 2002; Ho et al., 2001; Kato et al., 2002; Ramesh et al., 1997) as well as SH2/SH3 adaptor proteins such as Grb2 (Carlier et al., 2000) and Nck (Rohatgi et al., 2001). By contrast, WAVE proteins assemble into macro-molecular complexes (Eden et al., 2002; Gautreau et al., 2004), which initiate Arp2/3 complex-dependent actin polymerization upon activation by the small Rho-family GTPase Rac1 (Innocenti et al., 2004). Thus, there is increasing agreement on the importance of WAVE proteins for Rac-induced formation of lamellipodia and peripheral or dorsal membrane ruffling (Innocenti et al., 2004; Steffen et al., 2004; Suetsugu et al., 2003; Yamazaki et al., 2003; Yan et al., 2003), whereas the precise cellular functions of WASP and N-WASP are less well understood. The latter have been implicated in the formation of podosomes (Jones et al., 2002) or invadopodia (Mizutani et al., 2002; Yamaguchi et al., 2005), T-cell antigen receptor signalling (reviewed by Badour et al., 2003) and in different types of endosome motility (Benesch et al., 2002; Taunton, 2001). Moreover, N-WASP has been proposed to be essential for receptor-mediated endocytosis (Gundelfinger et al., 2003; Kessels and Qualmann, 2002). Indeed, N-WASP can interact directly with several proteins implicated in endocytosis, e.g. syndapin (also named PACSIN) proteins (reviewed by Kessels and Qualmann, 2004), intersectin-1 (Hussain et al., 2001) and indirectly with the large GTPase dynamin (reviewed by Engqvist-Goldstein and Drubin, 2003). The latter association could be mediated for instance by Nck, Grb-2 or a novel protein known as Tuba (Salazar et al., 2003), all of which share the capability of directly interacting with both N-WASP and dynamin via independent interaction surfaces (reviewed by Schafer, 2004).

In a seminal study, Merrifield et al. (Merrifield et al., 2002) simultaneously imaged actin and clathrin at the ventral plasma membrane of live cells using time-lapse evanescent field or total internal reflection (TIRF) illumination (reviewed by Toomre and Manstein, 2001) to reveal transient bursts of actin accumulation, which frequently coincide with the disappearance of clathrin-coated structures (CCS), most presumably single clathrin-coated pits. By combining TIRF microscopy (TIRFM) with conventional epifluorescence microscopy, evidence was provided that the disappearance of these clathrin-coated structures, as seen by TIRF illumination, most probably reflects the dissociation of clathrin-coated vesicles and their movement away from the plasma membrane out of the zone of TIRF illumination, which only penetrates 50-100 nm above the surface of the coverslip (Merrifield et al., 2002; Rappoport et al., 2004). Interestingly, both N-WASP and the Arp2/3 complex also accumulate at sites of clathrin pit endocytosis (Merrifield et al., 2004), although the relevance of their recruitment remained unknown.

We have extended this approach not only to directly follow the endocytosis of the epidermal growth factor (EGF) receptor and molecules established in EGF receptor signalling, as well as a number of proteins associated with the N-WASP/Arp2/3 complex actin polymerization machinery, but also to carefully evaluate the consequences of N-WASP loss of function for actin assembly. Using cell lines derived from N-WASP knockout mice, our results establish a significant contribution of this protein to the frequency of formation of this novel actin domain, and suggest a potential link of this function to the efficiency of EGF receptor internalization.

Materials and Methods

Expression constructs

mRFP-clathrin was generated by cloning human clathrin light chain A cDNA (kindly provided by Ernst Ungewickell, Hannover, Germany), using XhoI and EcoRI restriction enzymes, into a pEGFP-C3 backbone vector (BD Biosciences, Palo Alto, CA, USA) driving the expression of mRFP (kindly provided by Roger Y. Tsien, La Jolla, CA, USA) instead of EGF. The expression construct for EGF-tagged human β-actin was purchased from BD Biosciences; mRFP-actin has been described previously (Pacholsky et al., 2004). Human ARPC5A cDNA was fused into pEGFP-C1 (BD Biosciences) using BgII and SalI. mRFP-tagged EGFR was generated by exchanging EGFP for mRFP in an EGFP-EGFR expression construct kindly provided by Donna Arndt-Jovin (Göttingen, Germany) (Brock et al., 1999). Constructs for EGF-tagged N-WASP, WASP, WAVE2, WIP, Nck1/2 and Grb2 were as described previously (Benesch et al., 2002; Lommel et al., 2001; Scaplehorn et al., 2002). Expression constructs for myc-tagged Scar-WA and Scar-W (Machesky and Insall, 1998) were kindly provided by Laura Machesky (Birmingham, UK); mRFP-WAVE2 was from Julia Ehinger (Braunschweig, Germany) and EGFP-Sos-1 (Innocenti et al., 2002) was kindly provided by Giorgio Scita (Milan, Italy).

Cells and transfections

N-WASP<sup>−/−</sup> and the respective parental cell line (N-WASP<sup>+/+</sup>) were grown as described previously (Lommel et al., 2001) and transfected using FuGENE6 (Roche Molecular Biochemicals, Mannheim, Germany) or METAFECTENE<sup>TM</sup> (Biontex Laboratories GmbH, Munich, Germany) essentially according to manufacturers’ instructions. For video microscopy, cells were allowed to spread for at least 5 hours on acid-washed glass coverslips coated with 50 μg/ml fibronectin (Roche). For the analysis of actin assembly during CCS internalization upon Arp2/3 complex sequestration, cells were transiently transfected on coverslips with a mixture of Scar-WA or Scar-W (as control) and mRFP-clathrin and EGFP-actin in a 2:1:1 ratio and subjected to time-lapse two-colour total internal reflection (TIRF) microscopy. Overexpression of the myc-tagged polypeptides was confirmed by western blotting employing monoclonal anti-myc antibodies (Clone 9E10, ATCC), and at the single cell level by immunolabelling of parallel samples with 9E10 antibodies followed by Alexa Fluor 350®-coupled goat anti-mouse antibodies. Prominent anti-myc staining was observed in 100% of the cells (n=63 for myc-WA and n=62 for myc-W) expressing mRFP-clathrin and EGFP-actin (not shown).
Total internal reflection (TIRF) microscopy
Live cells were maintained on Olympus IX-70 or Zeiss Axiovert 200 inverted microscopes with open heating chambers (Warner Instruments, Hamden, CT or PeCon GmbH, Erbach, Germany) at 32°C. Dual-colour through-the-objective TIRF microscopy was performed by simultaneous illumination with the 488 and 568 lines from argon/krypton ion lasers (Innova 90, Coherent, UK or Melles-Griot, Carlsbad, CA, USA), individually tuned with acousto-optical tunable filters and coupled into single-mode optical fibres. TIRF condensers from TILL photonics (Gräfeling, Germany) and Visitron Systems (Puchheim, Germany) were mounted on Olympus TIRF condensers from TILL photonics (Gräfeling, Germany) and performed by simultaneous illumination with the 488 and 568 lines 32°C. Dual-colour through-the-objective TIRF microscopy was performed using Metamorph software, and fluorescence intensities were measured by adding a 100-fold excess of cold EGF.

Statistical analyses and quantifications
Quantification of the recruitment of components of the actin polymerization or EGFR signalling machineries to internalizing clathrin-coated structures as shown in Fig. 1D and Fig. 4D was performed as follows. Briefly, single pit-like structures containing mRFP-tagged clathrin light chain A, identified essentially as described previously (Merrifield et al., 2002), and disappearing during the time-course of TIRF video microscopy, were manually assessed for the recruitment of the respective component visualised in the EGFP channel and counted. For each cell, three randomly selected regions with an area of approx. 20 µm² were examined throughout the entire time course of the time-lapse recording. For each component, a total of at least 100 individual internalization events was analysed. The results were displayed as mean percentages ± standard errors of means (s.e.m.) of positive recruitments derived from at least eight independent cells. Statistical analyses were performed using Microsoft Excel 2000, Sigma Plot 8 and Minitab 10.5 software.

For assessing average peak intensities of clathrin or actin fluorescence prior to internalizations as shown in Fig. 2C, circles of 0.74 µm diameter were drawn around individual pit-like structures using Metamorph software, and fluorescence intensities were determined over a time period of approx. 16 seconds (corresponding to nine video frames) prior to pit disappearance. For each internalization event and component, average peak accumulation was defined as the arithmetic mean of the three frames of maximal fluorescence. For each processed frame, background fluorescence was determined over a time period of approx. 16 seconds (corresponding to nine video frames) prior to pit disappearance. For each component, average peak accumulation was defined as the arithmetic mean of the three frames of maximal fluorescence. For each processed frame, background fluorescence was determined over a time period of approx. 16 seconds (corresponding to nine video frames) prior to pit disappearance. For each component, average peak accumulation was defined as the arithmetic mean of the three frames of maximal fluorescence. For each processed frame, background fluorescence was determined over a time period of approx. 16 seconds (corresponding to nine video frames) prior to pit disappearance. For each component, average peak accumulation was defined as the arithmetic mean of the three frames of maximal fluorescence.

Internalization studies
EGF was purchased from Intergen, Rhodamine-EGF from Molecular Probes and 125I-EGF from Amersham Biosciences. Internalization assays of rhodaminated ligand or 125I-EGF were performed essentially as described previously (Haglund et al., 2003). For immunofluorescence, cells were grown on glass coverslips, serum starved for 3 hours, stimulated with Rhodamine-EGF (1 µg/ml) for 10 minutes at 37°C, washed once with PBS, fixed in 4% formaldehyde in PBS for 10 minutes and finally counterstained with DAPI.

For internalization assay with the 125I ligand, cells grown on 24-well dishes were serum starved for 3 hours prior to the addition of 1.5 ng/ml of 125I-EGF in binding medium (DMEM with 20 mM Hepes, pH 7.4 and 0.1% BSA) at 37°C. After the indicated times, cells were washed three times with cold DMEM to remove unbound ligand and then incubated with 0.2 M acetic acid (pH 2.8) containing 0.5 M NaCl at 4°C for 5 minutes to determine the amount of surface-bound 125I-EGF. Finally, the cells were lysed in 1 M NaOH to measure internalized radioactivity. The rate of internalization is expressed as the ratio between internalized and surface 125I-ligand for each time point. The data were statistically compared using linear regression analysis. Low concentrations of 125I-EGF were used to avoid saturation of the internalization machinery. Non-specific binding was measured by adding a 100-fold excess of cold EGF.

Results
Efficient actin and Arp2/3 complex accumulation at clathrin-coated pits is dependent on N-WASP
In exciting recent studies, Merrifield et al. proved the feasibility of directly visualizing the transient recruitments to clathrin-coated structures of actin or of associated proteins by dual-colour time-lapse TIRF microscopy (Merrifield et al., 2002; Merrifield et al., 2004). We employed this technique to directly test the role of the nucleation-promoting factor N-WASP in the assembly of this actin domain and for the recruitment of the Arp2/3 complex to these structures. We have recently characterised fibroblast cell lines from conditional N-WASP knockout mice (N-WASPdel/del) and their respective precursors (N-WASPflox/flox) (Benesch et al., 2002; Lommel et al., 2001), which were transiently co-transfected in this study with clathrin light chain A fused to monomeric red fluorescent protein (Campbell et al., 2002) (mRFP-clathrin) and with GFP-tagged actin, N-WASP or the Arp2/3 complex subunit p16 (also known as ARPC5, isoform A), respectively. The correct incorporation of mRFP-clathrin was confirmed by comparison with immunolabelling of clathrin heavy chain (not shown). Both revealed the same homogenous distribution of clathrin-coated structures (CCS) at the plasma and Golgi membranes. When imaging the control cell line (N-WASPflox/flox) by TIRFM, actin, the p16 subunit of the Arp2/3 complex and N-WASP transiently accumulated as distinct spots at the plasma membrane, which commonly overlapped with CCS. In order to examine the frequency of accumulation of cytoskeletal proteins during invagination and fission of single clathrin-coated pits, we focused on small CCS, which abruptly disappeared within the duration of time-lapse recording. Fig. 1A-C shows examples of transient accumulations of actin, p16 and N-WASP shortly before and during the disappearance of single clathrin-coated structures in control fibroblasts, which are similar in intensity and duration to the dynamics previously described for Swiss 3T3 cells (Merrifield et al., 2002; Merrifield et al., 2004). Representative movies showing transient accumulations of actin or N-WASP, both of which
disappear simultaneously with clathrin can be seen in the supplementary material (Movies 1-2).

To test the proposed function of N-WASP in the assembly of this type of actin structure, we quantified the number of pits recruiting these cytoskeletal proteins during disappearance in both control and N-WASP knockout cells (Fig. 1D).

Interestingly, the accumulation of both actin and the Arp2/3 complex (also see Fig. S1 in supplementary material) was reduced by approximately 50% in the absence of functional N-WASP (Fig. 1D). Nevertheless, about 40% and 70% of CCS disappeared without any detectable actin assembly in control and N-WASP knockout cells respectively, confirming previous results showing a non-obligatory role of actin assembly for endocytosis in mammalian cells (Fujimoto et al., 2000; Merrifield et al., 2002). Since the absence of functional N-WASP reduced the population of actin-positive and Arp2/3-complex-positive disappearing CCS only by half, we wondered whether those CCS capable of actin recruitment in control cells constitute two functionally distinct subpopulations, one N-WASP dependent and the second N-WASP independent. This scenario would be expected to correlate with only an approximately 25-30% recruitment frequency of N-WASP to disappearing CCS. Alternatively, although not obligatory, the presence of N-WASP might just function to increase the efficiency of actin assembly on all pits capable of recruiting the actin polymerization machinery, which would require N-WASP dynamics similar to actin or Arp2/3 complex. To distinguish between these possibilities, we quantified the frequency of EGFP-N-WASP recruitment to disappearing CCS (Fig. 1D). This value was very similar to actin and Arp2/3 complex recruitment frequencies in control cells.

Fig. 1. N-WASP deficiency causes decreased actin and Arp2/3 complex recruitment to internalizing CCS. (A-C) Internalization of individual CCS (red in merged images) in control (N-WASP⁺⁄⁺) fibroblasts can coincide with transient recruitment to these sites (arrowheads) of actin (A), Arp2/3 complex (p16 subunit; B) or N-WASP (C) (each green in merged images) as revealed by TIRFM. As described previously (Merrifield, 2004), the transient recruitment of each component to pre-existing clathrin-coated structures is followed by simultaneous disappearance of both clathrin and the respective component from the plane of TIRF illumination. Time is given in seconds; scale bar: 1 μm.

(D) Quantification of recruitment of the respective components to internalizing clathrin-coated structures. All proteins were co-expressed as GFP-tagged proteins with mRFP-tagged clathrin light chain A in precursor (floxFloX) or N-WASP-defective (del/del) fibroblasts as indicated. Numbers give recruitment during CCS internalization (as a percentage) for each ectopically expressed protein. Note the significant reduction in recruitment frequencies of both actin (27.9±2.7%, n=15, 224 events) and Arp2/3 complex (p16: 24.1±4%, n=22, 151 events) in del/del cells as compared to parental controls (actin: 57.4±2.7%, n=14, 221 events; p16: 73.1±3%, n=24, 257 events). Differences between control and del/del cells were confirmed to be statistically significant using a non-parametric Mann-Whitney Rank Sum test (P<0.00001). The recruitment frequency of EGFP-N-WASP (floxFloX: 64±5.7%, n=8, 116 events; del/del: 58.8±5.4%, n=8, 107 events) was independent of the presence of endogenous protein and similar to those scored for actin and Arp2/3 complex in parental precursor cells (floxFloX).
N-WASP and clathrin pit internalization

(approximately 60%), demonstrating that the remaining actin recruiting structures in N-WASP knockout cells did not reflect a distinct subpopulation of CCS. This conclusion was corroborated by the observation, using TIRFM, that cells co-expressing actin and N-WASP displayed patterns of spot-like accumulations at the ventral plasma membrane, which were virtually identical for both components (see Fig. S2 in supplementary material). As expected, the presence of endogenous N-WASP did not change the recruitment frequency of the ectopically expressed GFP-tagged protein (Fig. 1D).

To test if actin accumulations, which coincided with the disappearance of CCS in N-WASP knockout cells, were in any way different from those in controls, we also quantified the relative intensities of actin accumulations at single clathrin-coated pits in both cell types (Fig. 2). Fig. 2A,B shows representative examples of CCS, which internalized without (A) or with coincident actin recruitment (B). Assessment of the relative accumulations of actin and clathrin in both control and N-WASP-defective cells revealed no significant differences in the average peak intensities of the respective components shortly before their internalization (Fig. 2C), proving that once actin assembly at CCS occurred in the absence of functional N-WASP, it was indistinguishable in duration (not shown) and intensity to actin accumulations elicited in control cells (Fig. 2C).

Recruitment of WASP and N-WASP-associated proteins to clathrin-coated pits

We have shown previously that N-WASP is essential for actin comet formation stimulated by increased cellular InsPtd(4,5)P$_2$ or phosphotyrosine levels in a pathway that involves SH2/SH3-adaptor proteins such as Nck and WIP family proteins (Benesch et al., 2002). Both Nck and WIP proteins are readily expressed in control and N-WASP-defective cells as assessed by western blotting (see Fig. S3 in supplementary material). A series of experiments, including recruitment analyses of these proteins in the presence and absence of functional N-WASP and of various WIP and Nck mutants, indicated that these proteins function upstream of N-WASP in InsPtd(4,5)P$_2$-induced vesicle movement (Benesch et al., 2002). Interestingly, Nck and InsPtd(4,5)P$_2$ can synergistically activate N-WASP- and Arp2/3-complex-mediated actin polymerization in vitro (Rohatgi et al., 2001).

InsPtd(4,5)P$_2$-induced actin comets – as induced by overexpression of phosphatidylinositol-4-phosphate 5-kinase (PtdIns4P 5-kinase/PIP5K) – are known to propel endosomal vesicles at their tips, which largely originate from both the trans-Golgi network (TGN) and the plasma membrane (Rozelle et al., 2000), and recruit the large GTPase dynamin (Lee and De Camilli, 2002; Orth et al., 2002). Moreover, simultaneous TIRF imaging of both actin and N-WASP co-expressed in untreated cells revealed occasional spontaneous formation of actin comet tails tipped by N-WASP within the plane of illumination, i.e. very close to the plasma membrane (not shown), suggesting that at least a fraction of such actin comets may indeed originate from the plasma membrane. Hence, it is reasonable to assume that the molecular machinery driving PtdIns4P 5-kinase-induced actin comet motility is similar to the one mediating actin assembly on clathrin-coated pits.
To test this hypothesis, we co-expressed clathrin with the N-WASP-associated proteins Nck1 and Nck2 and WIP in both control and N-WASP-defective cells and followed their dynamics employing TIRFM during CCS disappearance. Nck1 (not shown), Nck2 and WIP (see Fig. S4 in supplementary material) transiently accumulated at CCS with similar duration and intensity to N-WASP, actin or Arp2/3 complex, indicating that these components may constitute a large protein complex, which can form during late stages of invagination, fission and/or departure of endosomal vesicles from the plasma membrane. Moreover, both types of proteins coincided with CCS internalization in the presence (see Fig. S4A in supplementary material) and absence (see Fig. S4B in supplementary material) of functional N-WASP. Interestingly, quantitative assessment of the recruitments of WIP and Nck2 to internalizing CCS not only revealed frequencies higher than those scored for actin and Arp2/3 complex in control cells (flox/flox), but also only slightly reduced incorporation into disappearing CCS in the absence of functional N-WASP (del/del) (see Fig. S4C in supplementary material). Together, these data show that association of Nck and WIP family proteins with CCS can be separated from N-WASP-mediated actin assembly, indicating a potential function in recruitment to and/or molecular regulation of the latter at these sites, similar to previous observations on the surface of InsPtd(4,5)P$_2$-induced endomembranes.

Interestingly, the haematopoietic WASP, which is absent in both control and N-WASP knockout fibroblasts and could substitute for N-WASP in driving PtdIns4P 5-kinase-induced vesicle movement in knockout cells (Benesch et al., 2002), also targeted to these structures upon ectopic expression (see Fig. S5 in supplementary material), demonstrating potential overlapping functions of N-WASP and WASP in amplifying actin accumulations at clathrin-coated pits.

Actin assembly at clathrin-coated structures is Arp2/3 complex dependent

The quantifications of actin and Arp2/3 complex association with internalizing CCS (Fig. 1D) indicated that actin polymerization at these sites is driven by Arp2/3-complex-mediated actin filament nucleation. To test this hypothesis directly, we employed an experimental sequestration of the Arp2/3 complex by overexpression of the WA-domain of Scar1 (WA), and as a control the same fragment lacking the Arp2/3-complex-binding acidic domain, but harbouring residues mediating interaction with monomeric actin (W) (Machesky and Insall, 1998). The WA-domain was successfully employed previously to inhibit lamellipodia formation (Machesky and Insall, 1998) and the actin-based motility of Listeria monocytogenes both in vitro and in vivo (May et al., 1999). To directly examine actin polymerization at CCS upon Arp2/3 complex inhibition, we co-transfected N-WASP$^{flox/flox}$ and N-WASP$^{del/del}$ cells with a mixture of EGFP-tagged actin, mRFP-clathrin and myc-tagged variants of either WA or W, followed by time-lapse dual-colour TIRF microscopy. Expression of the respective myc-tagged proteins was confirmed as described in Materials and Methods. Interestingly, in both cell types (see Fig. 3 for N-WASP$^{flox/flox}$ and not shown), WA-expressers internalized clathrin pits without any detectable coincident actin polymerization, while cells expressing the W-domain showed actin accumulations similar in intensity and

Fig. 3. Actin assembly at CCS is Arp2/3 complex dependent. TIRFM images of N-WASP$^{flox/flox}$ fibroblasts transiently co-expressing mRFP-clathrin light chain A (red in merged), EGFP-actin (green in merged images), and either WA-Scar1 or W-Scar1. Note the disappearance of CCS in cells expressing WA without any detectable coincident actin polymerization (asterisks) and the apparent actin assembly at disappearing CCS in the W-expressing control (arrowheads). Time is given in seconds; scale bar: 1 µm. The quantifications (bottom) of actin recruitments during CCS internalization revealed a complete abolition upon WA over-expression (0%, n=16, 110 events) and levels comparable to wild-type cells (Fig. 1D) in W-expressers (69.9±2.7%, n=16, 223 events).
duration to wild-type cells (Fig. 3 and Movie S3 in supplementary material). Moreover, quantification of the recruitment frequencies of actin to internalizing CCS in both cell populations (at least 16 movies from three independent experiments for each population) revealed the complete abolition of actin assembly upon Arp2/3 complex sequestration. Indeed, there was not a single actin accumulation event scored as opposed to close to a 70% recruitment frequency in cells co-expressing the W-domain (Fig. 3). The lack of decrease in actin accumulation frequency upon W-domain expression proves that the abolition of actin assembly at CCS by WA cannot be explained by depletion of the monomeric actin pool. Instead, these data strongly suggest that actin accumulation at CCS depends on proper subcellular positioning of the Arp2/3 complex. They also imply that actin assembly at these sites is exclusively driven by de novo polymerization and not by recruitment of filaments, and corroborate recent observations suggesting that effects on endocytosis are mediated by a dynamic actin cytoskeleton (Yarar et al., 2005).

The fact that N-WASP knockout cells lacking both functional N-WASP and the haematopoietic WASP were still capable of actin and Arp2/3 complex accumulation at these sites, albeit in a reduced fashion, prompted us to explore if WAVE proteins, the other subfamily of the WASP/WAVE family, may be responsible for driving the remaining Arp2/3-complex-dependent actin assembly. Western blot analyses revealed that both N-WASP<sup>W</sup> and N-WASP<sup>W</sup> cells expressed significant amounts of WAVE2, the ubiquitous WAVE isoform, but no detectable levels of WAVE1 and 3 (not shown), which are primarily expressed in brain (Sossey-Alaoui et al., 2003).

However, despite several attempts, we failed to detect any accumulation at CCS of an EGFP-tagged WAVE2 construct (not shown), which did, however, accurately target to the tips of lamellipodia upon Rac activation (Steffen et al., 2004). Since N-WASP, Arp2/3 complex and actin assembled at the same frequencies in control cells (Fig. 1D) and since co-expression of N-WASP with actin revealed a virtually identical pattern of spot-like accumulations at the plasma membrane (see Fig. S2 in supplementary material), the additional Arp2/3-complex-activating factor that was stripped by genetic N-WASP deletion would have to co-assemble with N-WASP. Therefore, to test directly for WAVE recruitment to internalizing N-WASP-containing structures, we also co-expressed a functional mRFP-WAVE2 construct (Stradal et al., 2004) with EGFP-N-WASP and explored whether disappearing N-WASP-containing structures contained any detectable WAVE2 accumulation. Although N-WASP-containing pit-like accumulations were at no time accompanied by any detectable WAVE2 recruitment (Fig. 4).

Finally, EGFP-tagged Sra1 (Specifically Rac-associated protein 1), now established as a constitutive component of the ubiquitous WAVE2 complex (Gautreau et al., 2004) and essential for lamellipodium protrusion (Innocenti et al., 2004; Steffen et al., 2004), also failed to target to this actin domain (not shown). Together, these data indicate that Arp2/3-complex-mediated actin assembly at clathrin pits is independent of WAVE complex function.

Absence of N-WASP reduces receptor-mediated endocytosis

The data described above demonstrated that the absence of functional N-WASP affects the frequency of actin accumulations during the invagination, fission and/or departure of single CCS. Moreover, it was proposed previously that N-WASP may serve an important function in receptor-mediated endocytosis (Hussain et al., 2001; Kessels and Qualmann, 2002). In order to test this directly, we measured the uptake of fluorescently coupled EGF in parental control and N-WASP knockout cell lines by examining intracellular fluorescence at different times after initiation of internalization. In these experiments, control and N-WASP knockout cell lines readily endocytosed fluorescently coupled EGF (Fig. 5A), demonstrating that the
lack of N-WASP does not generally block receptor-mediated endocytosis. In order to test if N-WASP loss of function quantitatively affected receptor-mediated endocytosis, we measured the initial rate of endocytosis using \(^{125}\text{I}\)-labelled EGF, which revealed a consistent reduction in the efficiency of EGF receptor endocytosis (Fig. 5B). This defect was specific for the lack of N-WASP, since the same assay yielded identical results when performed with a second, independently generated control and N-WASP knockout cell line pair (Lommel et al., 2001) (not shown). These data suggest that, although not essential, N-WASP-triggered actin assembly optimizes ligand internalization by receptor-mediated endocytosis.

N-WASP deficiency does not alter the association of EGFR signalling components with clathrin-coated structures

The absence of functional N-WASP caused a decrease in EGF internalization, which apparently coincided with reduced frequencies of actin accumulations at clathrin-coated pits. This observation raised the question whether the absence of N-WASP may affect the frequency or duration of accumulation of the EGF receptor and of components of the EGFR signalling pathway at CCS during internalization. Interestingly, efficient clathrin-dependent EGFR internalization requires the SH2/SH3-adaptor protein Grb2 (Huang et al., 2004; Jiang et al., 2003), which can directly activate N-WASP-mediated Arp2/3 complex activation and actin polymerization in vitro (Carlier et al., 2000). Abrogation of EGFR internalization upon depletion of Grb2 by RNA interference appeared to coincide with a reduction in EGFR localization at CCS (Jiang et al., 2003). Moreover, it has been suggested previously that actin filaments function in clathrin-mediated endocytosis, as scaffolds for efficient assembly of the endocytic machinery (Engqvist-Goldstein and Drubin, 2003) or by facilitating maturation of endocytic platforms (Da Costa et al., 2003). Importantly, initial comparisons of N-WASP and EGFR dynamics, using TIRFM on cells co-expressing GFP-N-WASP and mRFP-tagged EGFR, revealed that internalization of EGFR-containing pit-like structures from the plasma membrane can indeed coincide with significant N-WASP recruitment (see Fig. S6 in supplementary material). We therefore examined the recruitment of EGFR, Grb2 or the guanine nucleotide exchange factor Sos-1 to internalizing CCS in both control and N-WASP knockout cells. Interestingly, we could readily observe the internalization of CCS that had accumulated EGFR, Grb2 and Sos-1 in both control cells (Fig. 6A-C) and in cells lacking functional N-WASP (Fig. 6D and Fig. S7 in supplementary material). Moreover, detailed quantifications of the number of CCS, the internalization of which was coincident with that of EGFR, Grb2 and Sos-1 revealed a frequency of association of these components with CCS that was indistinguishable between control and N-WASP knockout cells (Fig. 6D), and hence independent of the frequency of actin accumulation at these sites. This conclusion was corroborated by the observation that the frequencies of association with CCS of the components of the EGFR signalling pathway were significantly higher (app. 80% or more) than those of components of the actin polymerization machinery. These data demonstrate that N-WASP-driven actin assembly is not required for efficient recruitment of EGFR and of its signalling associates to CCS, proving that the observed reduction of EGFR internalization cannot be explained by a vast alteration of EGFR distribution within the plasma membrane, but rather by a general reduction in the efficiency of clathrin-mediated endocytosis.

Discussion

Arp2/3-complex-mediated actin filament assembly is essential for various cellular processes (May, 2001; Millard et al., 2004), but the precise cellular functions of the respective nucleation promoting factors capable of activating the nucleating activity of Arp2/3 complex such as WASP and WAVE subfamily proteins are only now emerging (Daly, 2004; Vartiainen and Machesky, 2004). Here, we provide an in depth analysis of the contribution of the ubiquitously expressed N-WASP to the transient bursts of actin polymerization, which frequently accompany the late steps of receptor-mediated endocytosis via the clathrin pathway. We show that genetic deletion of N-WASP causes a significant reduction, but not abolition of actin

Fig. 5. N-WASP deficiency causes a reduction of EGF internalization. (A) After 3 hours of starvation, cells were incubated with rhodamine-EGF at 37°C for 10 minutes and directly processed for fluorescence microscopy. Red, rhodamine-EGF; blue, DAPI nuclear staining. (B) After 3 hours of starvation, cells were incubated with 1.5 ng/ml of \(^{125}\text{I}\)-EGF at 37°C for the time points indicated. The amount of surface and internalized radioactivity was determined at the end of the incubation. After correction for non-specific binding, the rate of internalization was expressed as the ratio between internalized/surface radioactivity (mean ± s.e.m. from three independent experiments). The differences were confirmed to be statistically significant (P<0.032 by linear regression analysis).
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Moreover, cells lacking functional N-WASP and WASP display reduced EGF internalization (Fig. 5), although the association of components of the EGFR receptor signalling machinery with clathrin appeared largely unaffected (Fig. 6), indicating a direct correlation between frequency of actin accumulation at CCS and efficacy of clathrin pit internalization. This view is reinforced by coinciding EGFR and N-WASP internalizations (Fig. S6 in supplementary material).

These observations have a number of important implications. In contrast to previous suggestions (Da Costa et al., 2003), N-WASP is not essential for the assembly, stabilization or maturation of clathrin-coated pits. Although our results support the conclusion that engagement of the actin cytoskeleton and Arp2/3 complex is not essential for endocytosis in mammalian cells (Fujimoto et al., 2000; Merrifield et al., 2002), they also add to the growing list of observations indicating an important contribution of actin reorganization in these processes. A number of actin interacting proteins have previously been implicated in receptor-mediated endocytosis. For instance, the Arp2/3 complex and dynamin interacting protein cortactin localizes to clathrin-coated pits and was proposed to drive the actin-mediated scission of clathrin pits from the plasma membrane (Cao et al., 2003; Zhu et al., 2005) (see also below). In addition, siRNA-mediated suppression of the actin filament and dynamin interacting adapter protein mAbp1 [mammalian actin binding protein 1, also known as SH3P7/Hip55 (Kessels et al., 2001)] was reported to abrogate receptor-mediated endocytosis (Mise-Omata et al., 2003), although it was not established whether ablation of this protein also reduced actin accumulation during this process. By contrast, suppression of Hip1R expression, the mammalian homologue of Sla2p, which is essential for endocytosis in yeast (Wesp et al., 1997), non-productively coupled the clathrin and actin machineries, leading to the formation of exaggerated actin structures coincident with defects in receptor-mediated endocytosis (Engqvist-Goldstein et al., 2004). Thus, both suppression and enhancement of actin polymerization may compromise receptor-mediated endocytosis, indicating that a finely tuned coupling of both machineries is required for efficient receptor-mediated endocytosis.

The generation of actin filaments has been proposed to function in at least three independent steps of clathrin-mediated endocytosis. Actin filaments were alternatively suggested to (i) serve as scaffolds for the assembly of endocytic machineries, (ii) generate forces aiding invagination and/or fission of clathrin-coated vesicles, or (iii) propel the formed vesicles into the cytosol (Engqvist-Goldstein and Drubin, 2003). The potential significance of the latter process is controversial and difficult to establish with the experimental setting employed.
here. A recent study employing drugs interfering with actin turnover and dynamics suggested a function in all the steps of clathrin-mediated endocytosis mentioned above (Yarar et al., 2005), although the timing of accumulation of actin and associated proteins observed here and by others (see Merrifield, 2004) indicates a particular contribution of actin assembly to late stages of invagination and/or fission (Fig. 1A-C; Fig. S4 in supplementary material) (Merrifield et al., 2002; Merrifield et al., 2004).

In an exciting recent study, Ehrlich et al. reported that early stages of clathrin pit assembly presumably include the formation of a low percentage of short-lived abortive structures (Ehrlich et al., 2004), possibly caused by a lack of stabilization by cargo capture or synchronous engagement of additional adaptors (Keyel and Traub, 2004). Interestingly, the same percentage (approx. 20%) of single pit-like structures was observed previously to disappear during TIRF illumination without detectable epifluorescence signal (Merrifield et al., 2002), demonstrating that the majority of CCS (80%) seen to disappear during TIRF microscopy represented productive internalization of clathrin-coated vesicles. This view is further corroborated by the fact that the average life-span and size before disappearance of the CCS examined here significantly exceeded those observed for the described abortive structures (Ehrlich et al., 2004; Keyel and Traub, 2004). Nevertheless, it is difficult at this stage to entirely exclude the possibility that decreased frequency of actin accumulation may cause a bias towards an increase in the number of abortive events.

As mentioned above, incubation of cells with different actin-perturbing drugs also caused a peculiar decrease of the numbers of newly formed CCS, at least at the ventral plasma membrane (Yarar et al., 2005). However, abolition of actin assembly by Arp2/3 complex sequestration, as performed here, did not block CCS formation (see Movie 3 in supplementary material and Fig. 3), although quantitative alterations may exist.

We have focused here on the molecular regulation of the actin assembly events most frequently observed during late stages of invagination and/or fission of individual CCS.

Our analyses imply that this type of actin assembly is an Arp2/3-complex-dependent process, which is regulated by WASP but not WAVE subfamily proteins (Figs 1, 3 and 4), and must include at least one additional Arp2/3-complex-activating factor to partly compensate for the absence of WASP/N-WASP in N-WASP knockout cells (Figs 1D, 2B). An attractive candidate protein is cortactin, which can activate Arp2/3-complex-mediated actin polymerization in the absence of a WASP protein in vitro (Urano et al., 2001; Weaver et al., 2001). Conversely, the relevance for this process of mAbp1 and Eps15, the yeast homologues of which, Abp1p and Pan1p, respectively, are also capable of independently activating the Arp2/3 complex (Duncan et al., 2001; Goode et al., 2001), is elusive.

It is worth mentioning in this context that the actin accumulations observed at some CCS in N-WASP knockout cells (Figs 1D, 2B) are, to our knowledge, the first cellular Arp2/3-complex-dependent structures that appear to lack any member of the WASP and WAVE families. Hence, it will be imperative to unravel the mechanism of their formation. In any case, our data emphasize the view that WASP and WAVE family proteins indeed drive the formation of distinct cellular actin structures (Stradal et al., 2004; Vartiainen and Machesky, 2004), for instance sites of endocytosis and peripheral protrusions such as lamellipodia and membrane ruffles, respectively, which we propose to be mediated, at least in part, by differential recruitment to these sites. The achievement of specificity for the respective sub-cellular location is not fully understood, but it is reasonable to assume that the robustness of differential targeting is mediated by engagement of these proteins in distinct protein complexes (Eden et al., 2002; Ho et al., 2004; Innocenti et al., 2004; Steffen et al., 2004) allowing for multiple specific interactions.

WASP and N-WASP can directly interact with a plethora of regulatory molecules like the Rho-family GTPase Cdc42, InsPtd(4,5)P2, WIP family proteins or SH2/SH3 adaptors such as Grb2 and Nck (reviewed by Millard et al., 2004; Stradal et al., 2004). Nck and WIP proteins are important for N-WASP recruitment in the actin-based motility of Vaccinia virus (Frischknecht and Way, 2001) and are recruited to the viral surface together as tri-molecular complexes with N-WASP. However, both protein families can target to InsPtd(4,5)P2-induced endomembranes independently of their interaction with each other and with N-WASP (Benesch et al., 2002). Likewise, these proteins robustly accumulate at disappearing CCS in both control and N-WASP-defective cells (Fig. S4 in supplementary material), and are therefore potentially contributing to N-WASP recruitment to CCS. The slight reduction of association frequencies during internalization scored for both WIP and Nck2 in N-WASP-defective cells (del/del; Fig. S4C in supplementary material) indicates that direct interactions of these proteins with N-WASP may stabilize them to a certain extent at CCS. However, as indicated above, N-WASP may be targeted to and activated at CCS by multiple interactions. At least three interaction surfaces of N-WASP were identified to recruit independently to InsPtd(4,5)P2-induced vesicles (Benesch et al., 2002). Interestingly, a recent study demonstrated that N-WASP mutants with increased valency for InsPtd(4,5)P2-binding induced spontaneous actin tail formation in cells, in a fashion that normally requires generation of InsPtd(4,5)P2 by PtdIns4P 5-kinase expression (Papayannopoulos et al., 2005). However, the relative relevance of InsPtd(4,5)P2 for recruitment and/or activation of N-WASP at CCS is currently unclear. Similarly, the Rho-family GTPase Cdc42 has also been implicated in N-WASP-dependent actin reorganizations (Ho et al., 2004; Rohatgi et al., 1999; Yamaguchi et al., 2005), and moreover in the regulation of EGF receptor trafficking (Wu et al., 2003).

Elucidation of a potential role of Cdc42 in N-WASP interactions for actin assembly initiated at these sites is an important goal for future investigations.

Besides affecting the efficacy of clathrin pit internalization, WASP protein-triggered actin assembly at clathrin-coated pits could also directly influence the elicitation and propagation of specific signalling pathways. Interestingly, T-cells from WASP knockout mice show a marked reduction in the coupling of T-cell receptor activation to induction of proliferation, apoptosis and interleukin-2 secretion (Zhang et al., 1999), which appeared to coincide with reduced endocytosis of the T-cell receptor (McGavin et al., 2001). Moreover, WASP and N-WASP bind to endocytic proteins of the intersectin family
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(Hussain et al., 2001; McGavin et al., 2001), the neuronal isoform of which (intersectin-1) displays upregulated GEF activity towards Cdc42 upon interaction with N-WASP in vitro, suggesting that the accumulation of N-WASP with endocytic proteins at CCS may indeed elicit focal activation of signalling. Although it is unknown whether actin polymerization affects the GTPase exchange activity of Sos-1, our data demonstrate that at least assembly and internalization of the EGFR-Grb2-Sos-1 complex occurs independently of frequent actin accumulation (Fig. 6). Thus, the challenge for the future will be to design experiments to directly correlate focal actin accumulations at CCS with determination of signal propagation from these sites in vivo. The combination of live-cell TIRF imaging and the analysis of cells deficient in actin polymerization and/or signalling machineries as described here paves the way towards a more detailed understanding of the intimate connection between actin assembly, endocytosis and signalling.

Conclusions

The internalization of clathrin-coated vesicles is frequently accompanied by transient bursts of actin assembly driven by the Arp2/3 complex. N-WASP and WASP, prominent activators of Arp2/3-complex-mediated actin filament nucleation, target to these sites and contribute to the frequency of formation of these actin structures. Cells lacking both WASP subfamily members show defects in actin assembly at clathrin-coated pits and reduced efficiency of EGF internalization. These data establish a distinct function of N-WASP/WASP in driving actin polymerization during receptor-mediated endocytosis and reinforce the view that actin assembly is not obligatory to, but can optimize clathrin-mediated endocytosis.

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