Development 130, 4427-4437

doi:10.1242/dev.00663

Kette regulates actin dynamics and genetically interacts with Wave and Wasp

Sven Bogdan and Christian Klämbt

Institut für Neurobiologie, Universität Münster, Badestr. 9, D-48149 Münster, Germany
*Author for correspondence (e-mail: klaembt@uni-muenster.de)

Accepted 12 June 2003

SUMMARY

During development of the *Drosophila* nervous system, *kette* is required for axonal growth and pathfinding. It encodes a highly conserved homolog of the Nck-associated protein 1 (NAP1) that genetically interacts with the *Drosophila* homolog of Nck, *dock*. We show that in vivo as well as in tissue culture models most of the Kette protein is found in the cytoplasm where it colocalizes with F-actin to which it can bind via its N-terminal domain. Some Kette protein is localized at the membrane and accumulates at focal contact sites. Loss of Kette protein results in the accumulation of cytosolic F-actin. The *kette* mutant phenotype can be suppressed by reducing the *wave* gene dose, demonstrating that *kette* antagonizes *wave* function. Overexpression of the wild-type Kette protein does not interfere with normal development, whereas expression of an activated, membrane-tethered Kette protein induces the formation of large F-actin bundles in both, tissue culture cells and in vivo. This gain-of-function phenotype is independent of *wave* but can be suppressed by reducing the *wasp* gene dose, indicating that Kette is able to regulate Wasp, to which it is linked via the Abelson interactor (Abi). Our data suggest a model where Kette fulfils a novel role in regulating F-actin organization by antagonizing Wave and activating Wasp-dependent actin polymerization.

Key words: F-actin, *Drosophila*, NAP1/Kette

INTRODUCTION

The actin cytoskeleton plays a central role in cell motility, morphology, phagocytosis and cytokinesis. It is spatially and dynamically reorganized, providing force for the shape change and surface movement in most eukaryotic cells (Borisy and Svitkina, 2000; Pantaloni et al., 2001). Rearrangement of actin is evoked rapidly by extracellular stimuli, and sets of actin-associated proteins are thought to act cooperatively in the polymerization, crosslinking and anchorage of actin filaments. Although the central dynamic nature of the actin system is known, actin organization and regulation are less well understood.

Actin dynamics crucially depend on the ability of the protein to switch from a monomeric (G-actin) to a filamentous form (F-actin). Polymerization of F-actin starts with the de novo nucleation of an actin trimer, a process that occurs relatively slowly and requires the action of the Arp2/3 complex (Machesky et al., 1994; May, 2001; Robinson et al., 2001; Welch et al., 1997). Subsequent elongation is fast and cells have to prevent spontaneous actin polymerization by expressing a variety of actin-binding proteins such as profilin (Schafer and Cooper, 1995). The nucleation activity of the Arp2/3 complex in turn is regulated by a set of activators, such as the members of the Wasp (Wiskott-Aldrich syndrome protein) and Wave (Scar – FlyBase) families (Higgs and Pollard, 2001; Suetsugu et al., 2002b; Takenawa and Miki, 2001). Wasp proteins are usually self-inhibiting and require small G proteins of the Rho family for activation (Kim et al., 2000; Miki et al., 1998a). In its activated GTP-bound form, Cdc42 can bind to the Crib (Cdc42/Rac Interactive Binding) domain of Wasp, releasing the auto-inhibition and thereby leading to the activation of the Arp2/3 complex (Higgs and Pollard, 2001; Rohatgi et al., 1999). By contrast, Wave, which does not bind Cdc42, is trans-inhibited through its association with members of the Kette (Hem – FlyBase) family, Sra1 (specifically Rac associated 1) and Abi (Abelson-interactor). Rac1 binding, presumably to Sra1, relieves the inhibitory function of this complex (Eden et al., 2002; Kobayashi et al., 1998; Miki et al., 2000).

Both, Cdc42 and Rac1 exert distinct functions in rearranging the F-actin cytoskeleton, GTP-bound Rac1 promoting lamellipodia and activated Cdc42 promoting filopodia formation (Hall, 1998). This matches the finding that Wasp enhances the generation of filopodia, whereas Wave activation near the cell membrane results in an increase of lamellipodia (Miki et al., 1998a; Takenawa and Miki, 2001).

Drosophila possesses only two genes, *wasp* and *scar*, that encode a Wasp and Wave homolog, respectively (Zallen et al., 2002; Ben-Yaacov et al., 2001). Both genes act largely independently of each other and have a strong maternal component. Only embryos lacking both the zygotic and the maternal gene functions display severe embryonic nervous system phenotypes. In the adult, *wasp* is required for formation of external mechanosensory organs, whereas *scar*/*wave* is – unlike *wasp* – required for normal formation of the compound eye. Recent structure function analyses demonstrated that *Drosophila* Wasp can perform at least part of its function independent of Cdc42 (Tal et al., 2002). Alternative modes of Wasp activation such as phosphorylation or SH3 (Src

homology 3) domain binding have been described (Cory et al., 2002; Scott et al., 2002; Suetsugu et al., 2002a).

Obviously, regulation of the dynamic F-actin cytoskeleton is pivotal especially during cell movement and thus requires a close link to the plasma membrane and guidance receptors involved in the perception of extracellular signals. The latter generally induce conformational changes in the cytoplasmic domain of membrane-anchored receptors, which recruits other proteins to the ligand-receptor complex. In the case of receptor tyrosine kinases (Rtk), autophosphorylation of tyrosine residues leads to the recruitment of SH2 domain containing adapter proteins. One of these is the SH2 SH3 adapter protein Nck, which is able to link several Rtks as well as guidance receptors like the Netrin-receptor DCC to the actin cytoskeleton (Li et al., 2001; Li et al., 2002). Nck can recruit additional proteins to the cell membrane via its three SH3 domains. Among the highly conserved Nck-interacting proteins are Wasp (Rivero-Lezcano et al., 1995), the non-receptor tyrosine kinase Abl (Adler et al., 2000) and Nap1 (Nck-associated protein 1), which is a member of the evolutionary conserved Kette family (Baumgartner et al., 1995; Hummel et al., 2000; Kitamura et al., 1996; Soto et al., 2002).

Based on its requirement for axonal growth, we have previously identified mutations in the *Drosophila kette* gene (Hummel et al., 2000). Loss of *kette* function primarily affects neurite growth and subsequently causes glial migration defects leading to a characteristic commissure phenotype in the embryonic CNS similar to the one observed in $wasp^{mat/zyg}$ mutants (Zallen et al., 2002) and *Drosophila* Nck (*dock*) mutants (Desai et al., 1999; Garrity et al., 1996). Furthermore, *kette* genetically interacts with mutations in the Nck homolog *dock* as well as with the small GTPase Rac1, supporting the notion that Kette may provide a novel mechanism linking extracellular signals to the actin cytoskeleton (Hummel et al., 2000). Recently, the Kette homolog Nap1 was found in a large 500 kDa protein complex comprising PIR121/Sra1, Abi, HSPC300 and Wave that keeps Wave in an inactive state in vitro (Eden et al., 2002).

However, to date it is still unclear how Kette could regulate the organization of the actin cytoskeleton in vivo. As Kette has been predicted to be an integral membrane protein with six transmembrane domains (Baumgartner et al., 1995), it might serve as a receptor recruiting Nck/Dock or Rho-GTPases to the membrane. We present biochemical and genetic evidence revealing that Kette is found predominantly in the cytosol. Only a small amount of Kette is recruited to the plasma membrane. In vivo as well as in tissue culture models, Kette protein colocalizes with F-actin and co-sedimentation assays revealed a direct interaction with F-actin. Within the membrane, Kette accumulates at the insertion sites of large F-actin bundles, suggesting that targeted localization of Kette may be required for its function. Loss of Kette protein leads to a Scar/Wave-dependent accumulation of F-actin within the cell. Ectopic expression of wild-type or different truncated Kette proteins in tissue culture cells or during *Drosophila* development does not affect F-actin formation or viability. However, expression of membrane-tethered Kette efficiently induces ectopic bundles of F-actin in a process depending on Wasp but not on Scar/Wave. These data indicate that Kette fulfils a novel role in regulating F-actin organization by antagonizing Wave and activating Wasp-dependent actin polymerization.

MATERIALS AND METHODS

Genetics

All crosses were performed at 25°C unless otherwise indicated. The following strains were used: $wasp^{1}$/TM6 $wasp^{3}$/TM6 (Ben-Yaacov et al., 2001); $scar^{k13811}$/CyO and $scar^{\Delta 37}$/CyO (Zallen et al., 2002); and $kette^{\Delta 2\text{-}6}$/TM6 and $kette^{J1\text{-}70}$/TM6 (Hummel et al., 2000). To determine the gain-of-function phenotype of $Kette^{Myr}$, we used the GAL4 system (Brand and Perrimon, 1993). Transgenic flies carrying the following constructs were generated following standard procedures: UAS-*kette*, UAS-$kette^{Myc}$; UAS-$kette^{1\text{-}374}$; UAS-$kette^{375\text{-}907}$; UAS-$kette^{908\text{-}1126}$ and UAS-$kette^{Myr}$. The $Kette^{Myr}$ construct was made by fusing DNA encoding the first 88 amino acids from *Drosophila Src1* (Simon et al., 1985) to the first codon of Kette. Several independent transgenic lines were analyzed in each case.

Two hybrid assay

To isolate proteins interacting with Kette protein, we inserted the *kette* as well as the *wasp* ORF in frame with the GAL4 DNA binding domain into the GBK-T7 vector (Clontech). With pGBK-Kette we screened a matchmaker *Drosophila* embryo cDNA library (1×10^6 clones tested) using the GAL4-based Two-hybrid System 3 from Clontech. pGAD-Abi contains the entire Abi-ORF.

Antibody production

The rabbit anti-Kette antibody (97/82), directed against a peptide derived from the middle region of Kette (652-666), KHFDDIRKPGDESYR, was made by Eurogentec (Belgium). In addition, polyclonal antibodies were generated against parts of Kette fused with a His_6-tag (Qiagen). pQE plasmids express amino acid regions 1-374, 375-906 and 907-1126 of Kette, respectively. Using these expression constructs, His_6-Kette fusion proteins were expressed in *E. coli* and purified with Ni-NTA resin (Qiagen) under denaturing conditions. Rabbits were immunized with purified proteins by Davids Biotechnologie (Germany).

Cell culture, transfection and immunofluorescence

Drosophila S2 cells were propagated in 1× Schneider's *Drosophila* media (Gibco) supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin in 75 cm^3 T-flasks (Sarstedt) at 25°C. For transfections, 5×10^5 cells were plated on glass cover slips (pretreated with fibronectin) in 24-well plates, cultured for 24 hours and transfected with Fugene 6 (Roche) at a transfection reagent:DNA ratio of 3:1. Cells were incubated 24 hours after transfection, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, stained with *Drosophila* anti-Kette antibody 97/82 (1:2000 dilution) followed by Alexa-fluorophore-conjugated goat anti-rabbit IgG antibody (Molecular Probes, 1:1000 dilution). For filamentous actin and nucleic acid staining, cells were prepared as described above and incubated in 1-2 units Alexa-fluorophore-conjugated phalloidin (Molecular Probes) and in 300 nM DAPI (Molecular Probes), respectively. Tyrosine phosphorylated proteins were detected using the PT-66 anti-phosphotyrosine antibody (Sigma). The samples were mounted in a 25% (w/v) Mowiol (Sigma) solution containing DABCO (Sigma), and visualized on a Leica LSM.

dsRNA production and RNAi treatment

570 bp of the *kette* ORF and 750 bp of the *wasp* and *scar/wave* ORF were inserted into the vector pLITMUS-28i (BioLabs) flanked by T7 promoters. After linearization, dsRNA was generated using a HiScribe RNAi Transcription Kit (BioLabs). dsRNA products were resuspended in water and annealed by incubation at 65°C for 30 minutes followed by slow cooling to room temperature and stored at –20°C. For RNAi treatment, 5×10^5 *Drosophila* S2 cells in 0.5 ml serum-free medium were plated on fibronectin coated cover slips in 24-well plates (Sarstedt). dsRNA (5 μg) were added directly to the media followed by vigorous agitation. The cells were incubated for

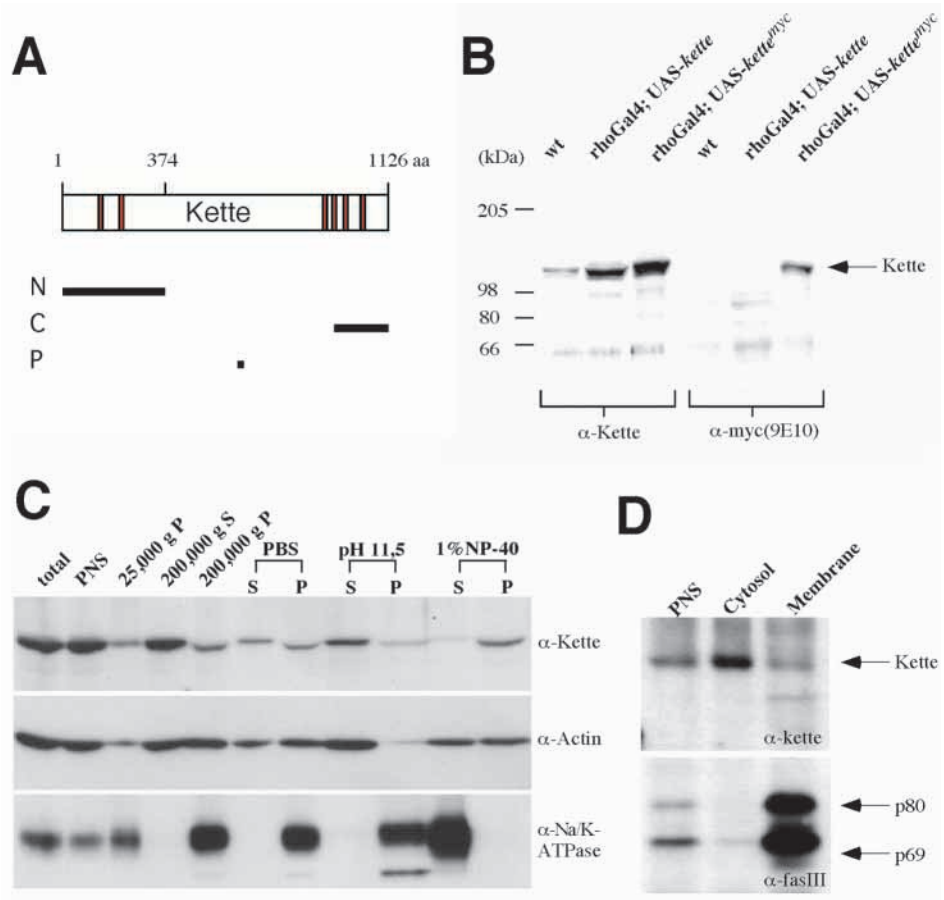


Fig. 1. Kette associates with membranes. (A) Schematic view of the Kette protein; hydrophobic domains are indicated. Different antisera were generated. N, N-terminal domain, amino acids 1-374; the M-serum directed amino acids 375-907 is not indicated; C, C-terminal domain, amino acids 908-1126; P, peptide antibodies, amino acids 652-666. (B) Western blot analyses of protein extracts prepared from wild-type, rhoGAL4/UAS-Kette full-length or rhoGAL4/UAS-Kette^{myc} embryos were probed either with the anti-Kette P antiserum or anti-Myc antibodies (Mab 9E10) as indicated. The Kette protein is ~120 kDa. Anti-Myc antibodies recognize a similar sized protein confirming the specificity of the antisera. (C) Differential centrifugation reveals that Kette is located primarily in the cytosol. Western blots were probed with anti-Kette antisera (top), anti-actin antibodies (middle) and anti Na^+/K^+ ATPase antibodies (bottom) to monitor a typical transmembrane protein. The different lanes show: total, total protein extracts of S2 cells; PNS, supernatant following 1,000 ***g*** centrifugation. The supernatant was subjected to 25,000 ***g*** centrifugation and subsequently to 200,000 ***g*** centrifugation. The pellet was re-suspended in either PBS at high pH or 1% NP-40 and subjected to 200,000 ***g*** centrifugation (S, supernatant; P, pellet). For each lane, equal amounts of total proteins were loaded on SDS-PAGE. (D) Post-nuclear supernatant (PNS) of wild-type embryos was subjected to equilibrium sedimentation on a discontinuous sucrose density gradient. Only small amounts of the Kette protein were detectable in the membrane fraction, while most of the Kette protein remains in high dense sucrose containing cytosolic proteins. Western blot analysis was performed using anti-Kette antisera (top) and anti Fas3 antibodies (bottom) to monitor a typical transmembrane protein. Equal amounts of total proteins in the post nuclear supernatant (PNS), membrane and cytosolic fraction were loaded for SDS-PAGE.

30 minutes at 25°C followed by addition of 1 ml medium containing 15% FCS. dsRNA treated cells were incubated for additional 2-3 days and analyzed by immunofluorescence and western blot analysis.

Actin binding assay

Actin was purified from rabbit skeletal muscle following the methods of Spudich and Watt (Spudich and Watt, 1971) and stored as G-actin at –172°C (Spudich and Watt, 1971). Actin was polymerized in a buffer of 100 mM KCl, 2 mM $MgCl_2$, 20 mM HEPES, pH 7.4, 0.5 mM β-mercaptoethanol and 2 mM NaN_3. Prior to use, MBP, MBP-Kette and actin were centrifuged at 200,000 ***g*** for 30 minutes in a tabletop ultracentrifuge (Beckmann Instruments) to pellet any aggregated protein. For binding assays, increasing amounts of full-length or truncated Kette proteins were incubated with 3 μM F-actin for 30 minutes at room temperature in polymerization buffer. The mixtures were centrifuged at 200,000 ***g*** for 30 minutes at 4°C. Equal amounts of supernatant and pellet were separated by SDS-PAGE followed by Coomassie blue staining.

Fractionation experiments

Fractionation of *Drosophila* embryonic and S2 cell extracts were performed as described (Zhang and Hsieh, 2000). The pellets were solubilized in SDS sample buffer and supernatants were precipitated in trichloroacetic acid, followed by solubilization in SDS sample buffer. Western blot analysis was carried out as described previously (Bogdan et al., 2001).

RESULTS

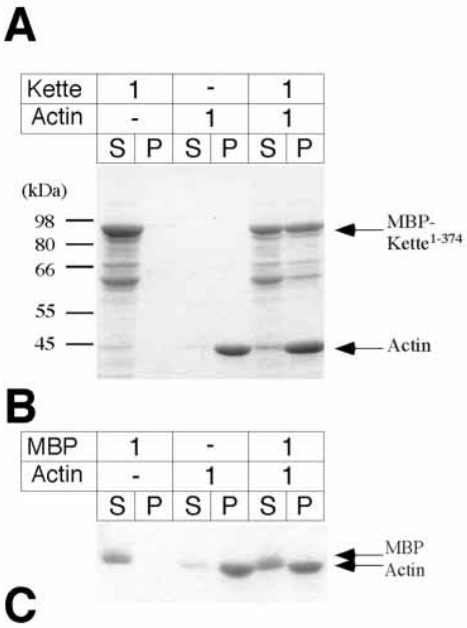
Kette is a cytosolic actin-binding protein

Kette was predicted to be an integral membrane protein (Baumgartner et al., 1995). To test this, we generated polyclonal antisera against the Kette protein (Fig. 1A). Western blot analyses were performed on protein extracts of wild-type embryos and embryos overexpressing the full-length Kette protein under the control of the GAL4 system. All antisera recognized a 120 kDa protein, which is in good agreement with the predicted molecular mass of 125 kDa for Kette (Fig. 1B). The specificity of the antisera was further supported by the detection of a recombinant Myc-tagged Kette protein recognized in vivo by both anti-Kette and anti-Myc antibodies (Fig. 1B) and RNAi experiments (see Fig. 4A). No evidence for post-translational proteolytic cleavage was obtained.

To examine whether Kette is an integral membrane protein, we first analyzed its distribution in *Drosophila* cell extracts prepared from embryos or *Drosophila* S2-tissue culture cells by differential ultracentrifugation and flotation assays (Fig. 1C,D). In both cases the results were identical. After ultracentrifugation at 200,000 ***g*** Kette was found predominantly in the cytosolic supernatant, whereas smaller amounts could be detected in the pellet containing membranes

and large protein complexes. The particulate fraction of Kette was efficiently extractable by sodium carbonate treatment (0.1 M, pH 11.5), but it was retained in the 1% NP-40 insoluble cytoskeletal fraction (Fig. 1C). To further investigate how much Kette protein really binds to membranes, a flotation assay was performed. We fractionated post-nuclear supernatants from *Drosophila* embryo lysates by isopycnic centrifugation on a discontinuous sucrose density gradient. Unlike the integral membrane protein FasIII (Patel et al., 1987), some of the Kette protein could float out of the most dense sucrose during centrifugation to equilibrium, while most of the Kette protein remains in high dense sucrose (Fig. 1D). Taken together, these data show that Kette is not an integral membrane protein but small amounts are associated with membranes.

The recovery of Kette protein in the 1% NP-40 insoluble fraction containing F-actin and other cytoskeletal proteins suggest an association between Kette and F-actin. To test this directly, we determined a possible interaction of Kette with F-actin in a co-sedimentation assay. Soluble, maltose-binding protein (MBP) fusion proteins containing full-length Kette or its N-terminal third (Kette$^{1-374}$) were generated. Both fusion proteins, and unaltered MBP as a control, were subjected to ultracentrifugation either alone or in a mixture with F-actin. Only the fusion proteins pelleted with F-actin (Fig. 2A,B), indicating that actin binding is mediated by the N terminus of Kette, which, however, lacks known F-actin binding sequence motifs. Furthermore, we identified three different actin isoforms (Act42A, Act5C and Act57B) as putative interaction partners of Kette in a yeast two-hybrid screen using full-length Kette as bait (Fig. 2C). Based on sequence analyses of these functionally redundant actin isoforms (Wagner et al., 2002), Kette is able to bind to the C-terminal 110 amino acids of G-actin.



Number of clones	Name	Protein length (aa)	Interaction region
2	Actin 5C	377	169/220 - 377
1	Actin 42A	366	220 - 360
1	Actin 57B	360	254 - 360

Fig. 2. Kette binds to actin. (A) Soluble Kette protein was prepared as a maltose-binding-protein (MBP) fusion and stayed in the supernatant (S) when centrifuged at 200,000 ***g*** for 30 minutes. By contrast, F-actin was found in the pellet (P). When soluble Kette is added to F-actin, about 50% of Kette associates with the F-actin. Proteins were separated by SDS-PAGE followed by Coomassie blue staining. (B) MBP does not bind to F-actin. (C) Kette interacts with G-actin. Four clones of highly related actin sequences were identified in a yeast two hybrid screen. Sequence analyses indicate that interaction with Kette occurs at the C terminus.

Subcellular localization of the Kette protein in vivo

Unfortunately, none of the antisera generated detects endogenous Kette protein in whole-mount preparations of wild-type embryos. As many epitopes are sensitive to methanol treatment required to prepare *Drosophila* whole-mount embryos, we studied the subcellular localization of the endogenous Kette protein in freshly dissociated cells from *Drosophila* embryos as well as in *Drosophila* S2 tissue culture cells (Fig. 3). In neuronal cells, which can be identified by expression of the HRP epitope (Jan and Jan, 1982), Kette is localized in the cytosol as well as in a punctuated pattern along the emerging neurites (Fig. 3A). In muscle cells, Kette is expressed in long fibers that resemble F-actin fibers. Indeed double staining experiments using phalloidin showed prominent co-localization with F-Actin (Fig. 3B). Both cell types are affected by the *kette* mutation (Hummel et al., 2000).

As shown by western blot analyses, *Drosophila* Schneider S2 tissue cells express high levels of Kette (Fig. 4A). When S2 cells are grown on fibronectin coated substrates, they adhere to the culture dish and adopt a characteristic pancake shape (Fig. 3C,D). The majority of the Kette protein is found in the cytosol around the nucleus; however, small amounts of Kette can be detected at the leading edge of lamellipodia-like extensions (Fig. 3C, arrowheads). Increased levels of Kette expression were noted on spoke-like structures extending from the nucleus to the membrane (Fig. 3C, arrows). Highest Kette concentration is generally found close to the cell membrane overlapping with tyrosine-phosphorylated proteins, which mark focal contact sites (Fig. 3D) (Parsons et al., 2000). F-actin is similarly distributed as Kette and the spokes showing high Kette expression correspond to thick F-actin bundles. At the cell margin, however, Kette appears to be localized closer to the membrane than the cortical F-actin network. In line with the above mentioned fractionation results this again indicates that some Kette protein is associated with the membrane.

Loss of *kette* function leads to an excess of F-actin in the cytosol

The above data showed that Kette can be detected at the membrane and it is able to bind to F-actin. First evidence for a regulatory function of Kette in F-actin organization stems from our phenotypic analysis of *kette* mutants (Hummel et al., 2000). Loss of *kette* function affects the organization of the F-actin cytoskeleton and is characterized by an excess of disorganized F-actin bundles. To better analyze the function of Kette in F-actin dynamics we reduced *kette* expression in *Drosophila* S2 cells by RNA interference (see Materials and Methods). As judged by western blot and immunofluorescence analyses, treatment of S2 cells with a 570 bp dsRNA fragment generated from the *kette*-coding region resulted in a marked

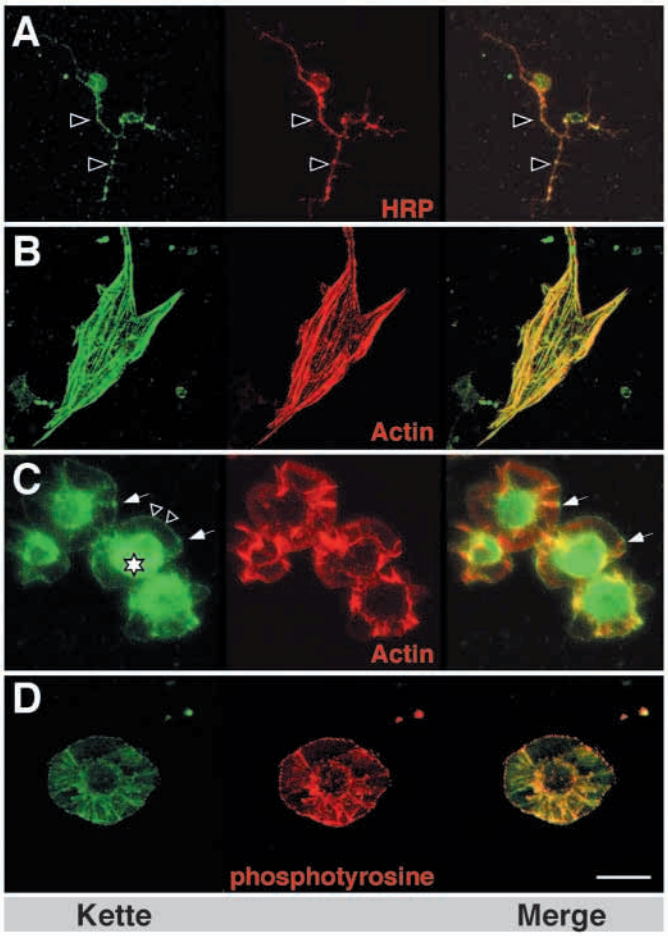


Fig. 3. Subcellular localization of endogenous Kette. (A,B) Primary tissue culture cells of *Drosophila* embryos were plated on fibronectin-coated cover slips and were allowed to differentiate over night. (C,D) Schneider S2 cells. Endogenous Kette protein was detected using anti-Kette antisera in a dilution of 1/1000. Anti-HRP antibodies recognize a carbohydrate present on all neuronal membranes. Anti-phosphotyrosine antibodies were used to detect focal contact sites. Phalloidin staining was used to visualize the F-actin cytoskeleton. A-C are projections of several confocal sections, D shows a single focal plane. Scale bar: 5 μm. (A) In neurons, Kette is expressed in an often punctuated pattern throughout the cell and can be detected in dendrites as well as axons. Higher levels of Kette are found at sites where the axon turns or branches (arrowheads). (B) Muscle cells are characterized by a highly regular F-actin cytoskeleton. Kette and F-actin expression largely overlap in these cells. (C) Schneider S2 cells endogenously express Kette. The majority of Kette is found in the perinuclear region (star). Small amounts of Kette are recruited to the leading edge of lamellipodia-like cell processes (arrowheads). Several Kette rich spokes extend from the nucleus to the membrane with higher levels of Kette at their end points close to the cell membrane (arrows). F-actin largely follows the Kette localization and can be detected surrounding the nucleus and in spokes extending to the membrane. In addition, a subcortical F-actin mesh can be detected in the lamellipodia-like structures just underneath Kette. (D) Kette expression as in C. Note the punctate appearance of Kette at the membrane. The distribution of tyrosine phosphorylated proteins frequently overlaps with Kette expression. Merged images are shown on the right.

reduction of Kette protein expression (compare Fig. 4A with Fig. 5A,B). No interference was seen with sense and antisense RNAs (data not shown). With increasing time after dsRNA treatment, cells with reduced levels of Kette show dramatic alterations in cell morphology and concomitant changes of the F-actin cytoskeleton. Within 3 days, cells appear to collapse and accumulate large amounts of F-actin. Two days after RNAi treatment, intense granular F-actin structures are observed which are similar to those in *kette*-null mutants (Hummel et al., 2000) (Fig. 5B, arrowhead).

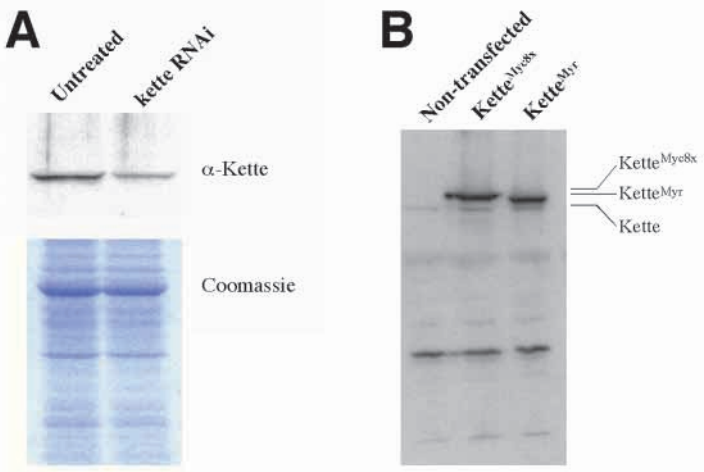


Fig. 4. Kette expression. (A) Top panel shows a western Blot probed for Kette expression (P-antiserum), equal amounts of protein were loaded on the gel (Coomassie stain). (B) Western blot showing Kette expression in S2 cells and similarly increased levels of Kette expression following transfection with *act5c*-GAL4 and UAS-*kette*^{Myc} or *act5c*-GAL4 and UAS-*kette*^{Myr}.

Kette represses Scar/Wave activity in vivo

These data led to the question how Kette influences actin dynamics. F-actin formation is controlled by the action of the Arp2/3 complex, whose activity is in turn regulated by members of the Wasp/Wave family. *Drosophila* has a single Wasp as well as a single Wave homolog. Recently, the Kette homolog Nap1 was found in a large 500 kDa protein complex comprising PIR121/Sra1, Abi and HSPC300 that keep Wave inactive in vitro (Eden et al., 2002). In agreement with this work disruption of *kette* function leads to an excess formation of cytoplasmic F-actin. In contrast to *kette* depletion, treatment of S2 cells with *scar* dsRNA leads to a reduced F-actin formation in the cytosol. Only some cortical F-actin is detected (Fig. 5C). Interestingly, disruption of Scar/Wave function resulted in a fairly uniform distribution of Kette in the cytosol and spoke-like F-actin structures cannot be recognized any more (Fig. 5C). This suggests an intact actin cytoskeleton is required to position Kette in the cell and may point to the functional relevance of the F-actin binding properties of Kette. Treatment of S2 cells with both *scar/wave* and *kette* dsRNA suppresses the excess formation of cytoplasmic F-actin induced by *kette* dsRNA alone. In all cell shape changes appeared slightly more severe compared with those evoked via *scar* dsRNA alone (Fig. 5C,D).

Further support for a Kette mediated repression of Scar/Wave activity stems from genetic analyses. Wild-type

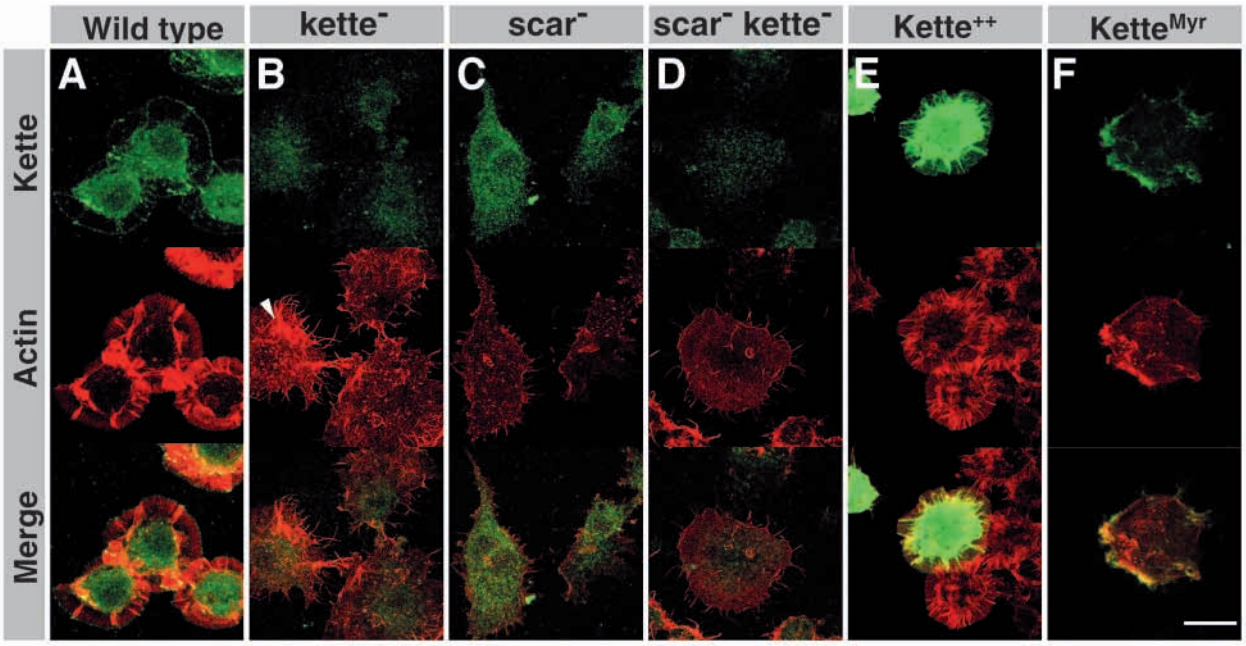


Fig. 5. Kette regulates F-actin organization. Kette expression (top), the F-actin cytoskeleton (middle) and the merge (bottom) are shown in Schneider S2 cells using anti-Kette antisera. All images represent stacks of confocal sections. (A) In wild-type cells, endogenous Kette protein largely colocalizes with F-actin. (B) Disruption of Kette expression by RNA interference leads to a concomitant disruption of the F-actin cytoskeleton. Forty-eight hours after treatment with *kette* dsRNA, aggregates of F-actin are found surrounding the nucleus (arrowhead). (C) Depletion of Scar/Wave by treatment with *scar/wave* dsRNA leads to a marked reduction in F-actin formation and alterations in cell morphology. Interestingly, in the absence of Wave, Kette appears to be uniformly distributed throughout the cell. (D) After RNAi for both *kette* and *scar/wave*, the formation of F-actin is not further reduced. (E) Overexpression of high levels of a Myc-tagged Kette protein does not lead to any changes in the organization of the F-actin cytoskeleton. The high dilution of the anti-Kette antiserum (1:50,000 compared with 1:2000 in A does not allow the detection of the endogenous Kette protein). (E) After expression of a membrane-tethered Kette protein, the F-actin cytoskeleton is rearranged. Large clumps of F-actin can be detected close to the membrane at sites that also show high levels of Kette expression. Scale bar: 5 μm.

embryos are characterized by clearly separated anterior and posterior commissures, which is due to the migrating midline glia (Fig. 6A). Embryos that lack zygotic *kette* function display a characteristic CNS phenotype and commissures appeared fused (Fig. 6B). By contrast, loss of zygotic Scar/Wave expression does not affect embryonic nervous system development (Zallen et al., 2002) (Fig. 6D). To test whether the *kette* mutant phenotype might due to an upregulation of *scar/wave* activity we removed one copy of the *scar/wave* locus in a *kette* background, which indeed suppressed the homozygous *kette* phenotype and distinct commissures are now recognizable (Fig. 6C). Taken together, these data suggest that in vivo Kette represses *scar/wave* activity.

Kette functions at the membrane

To further understand how Kette influences the dynamics of the F-actin cytoskeleton, we first conducted overexpression studies. In contrast to the *kette* loss of function phenotype,

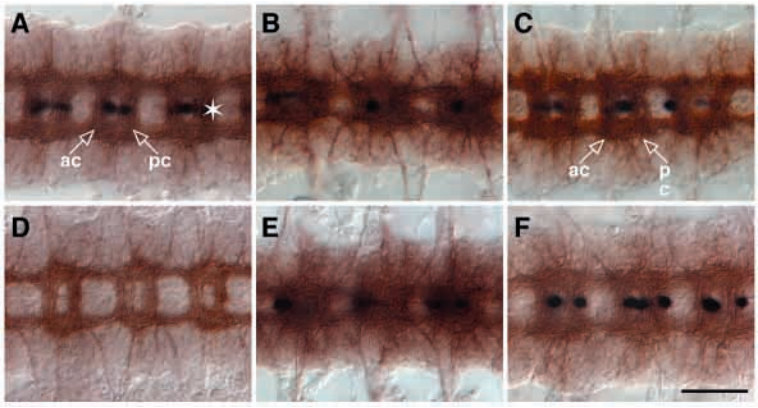


Fig. 6. *kette* antagonizes *scar/wave* function. Frontal views of embryonic nerve cords of stage 16 embryos. Axon tracts are labeled using Mab BP102 and HRP immunohistochemistry (brown), midline glia cells are labeled in blue (enhancer trap insertion *AA142*). (A) In a wild-type nerve cord, two commissures are found in each neuromere (anterior commissure, ac; posterior commissure, pc). They are clearly separated by the midline glia (star). (B) In homozygous mutant $kette^{C3\text{-}20}$ embryos the segmental commissures are not separated into distinct axon bundles and instead appear fused. (C) Removal of one copy of the *scar/wave* gene in a homozygous mutant $kette^{C3\text{-}20}$ embryo significantly restores CNS development and commissures are recognizable as distinct axon bundles. (D) Homozygous mutant *scar/wave* embryos display no mutant CNS phenotype. (E) Reduction of *wasp* function in a *kette* mutant embryo (wasp kette double mutant) does not modify the mutant *kette* phenotype. (F) Expression of a membrane-tethered Kette protein can rescue the *kette* mutant phenotype. A *rho*-GAL/UAS-$kette^{Myr}$; $kette^{C3\text{-}20}$/$kette^{C3\text{-}20}$ embryo is shown. Scale bar: 30 μm.

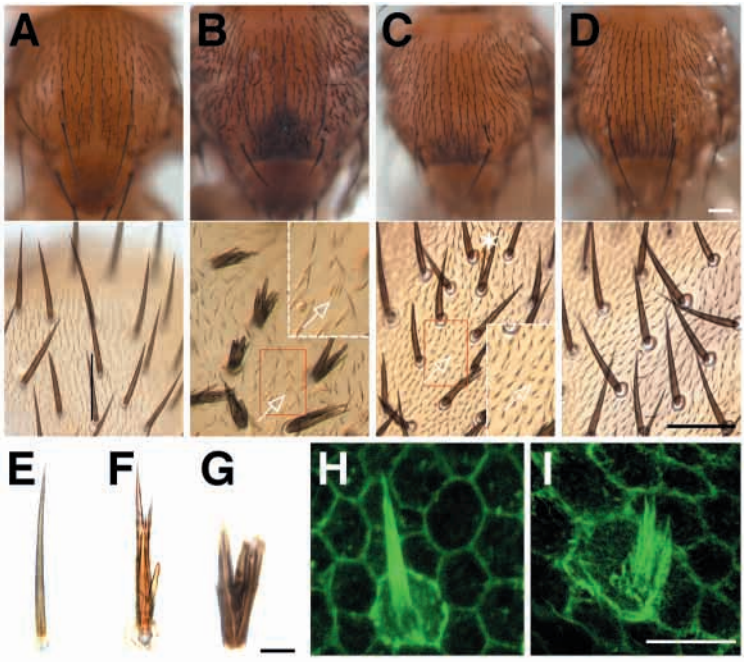


Fig. 7. Kette regulates F-actin formation in vivo. (A-D) Top row, dorsal view of a *Drosophila* notum. Bottom row, higher magnification showing the morphology of microchaete and epidermal hairs (scale bar: 10 μm). (A) Wild-type flies are characterized by an ordered array of macro- and microchaete, which are normally thin, straight and with a pointed end (E). All epidermal cells generate a small hair. (B) After expression of three copies of a UAS-Kette^{Myr} transgene in the *scabrous* pattern, bristle morphology is severely disrupted. Kette^{Myr} expression results in shorter, branched and thicker bristles (G). In addition, epidermal cells generate more than one hair (arrow). The area boxed in red is shown enlarged. (C) After expression of two copies the UAS-Kette^{Myr} transgene in the *scabrous* pattern results in a weaker phenotype; however, bristles are still forked and shorter (star and F) and epidermal cells develop more than one hair (arrow). The area boxed in black is shown enlarged. (D) Same genetic background as in C but lacking one copy of the *wasp* gene. The bristle phenotype evoked by Kette^{Myr} expression is suppressed. (H) During pupal development, bristle morphology is prefigured by an apical F-actin extension. (I) After Kette^{Myr} expression (three copies), F-actin formation is initiated in a broad region of the apical cell surface. In addition, the F-actin at the cell boundary appears to have a fuzzier organization after Kette^{Myr} expression. Scale bars: 100 μm in A-D; 2 μm in E-G; 10 μm in H,I.

overexpression of full-length protein as well as the expression of several deletion constructs did not lead to any phenotype in wild-type flies or S2 cells (Fig. 4B, Fig. 5E; data not shown). As the overexpressed Kette protein was found in the cytosol, it appeared likely that membrane-recruitment of Kette is crucial for its function. In addition, biochemical as well as genetic data suggest that Kette – like the human homolog Nap1, which binds to the membrane-associated SH2SH3 adapter NCK/Dock – acts at the membrane (Hummel et al., 2000; Kitamura et al., 1996).

To test this possibility directly, we generated a membrane-tethered form of Kette by fusing a myristyolation signal derived from the *Drosophila* Src1 protein to the N terminus of Kette (Kette^{Myr}). Overexpression of comparable amounts of Kette^{Myr} in S2 cells also led to a dramatic rearrangement of the cytoskeleton and F-actin accumulates close to the membrane at sites of Kette expression (Fig. 4B, Fig. 5F). Thus, membrane tethered Kette protein is able to induce local actin polymerization.

In order to assess whether the membrane-tethered Kette protein performs similar to the wild-type protein we established UAS-*kette^{Myr}* transgenic flies and conducted genetic rescue experiments. Expression of Kette^{Myr} in the *rhomboid* pattern in all CNS midline cells of mutant *kette* embryos suppresses the mutant phenotype in such that the segmental commissures can be clearly identified again which is not possible in mutant *kette* embryos (Fig. 6F). This indicates that the Kette^{Myr} protein performs wild-type functions.

Increased expression of membrane-tethered Kette reorganizes the actin cytoskeleton

Elevated expression of the activated, membrane-tethered, Kette protein is able to induce a dominant phenotype in a dose-dependent manner. Flies carrying a *scabrous*-GAL4 driver (Klaes et al., 1994) and three copies of the UAS-Kette^{Myr} transgene are characterized by pronounced alterations in bristle morphology (Fig. 7). Whereas in wild-type flies macrochaete and microchaete form as thin and relatively straight cuticular structures, expression of Kette^{Myr} leads to thicker and often forked bristles (Fig. 7A,B,E,G). In addition to the bristle phenotypes we observed defects in the epidermal hairs. In the wild-type, each epithelial cell will develop one fine hair, whereas Kette^{Myr} expression results in multiple hairs emerging from one cell (Fig. 7B,C, arrows). When we expressed only two copies of the Kette^{Myr} transgene, bristle development and epidermal hair formation was affected more moderately (Fig. 7C,F).

The bristle and hair phenotypes induced by Kette^{Myr} expression, reflect alterations in the organization of the apical F-actin cytoskeleton during pupal development when apical F-actin bundles prefigure bristles (Fig. 7H). In flies overexpressing Kette^{Myr}, a rearrangement of the F-actin cytoskeleton is apparent. In contrast to wild type, large and irregular F-actin bundles emerge. In addition, cell morphology is slightly altered, cells exhibit more irregular shapes and the cortical F-actin network appears fuzzy (Fig. 7I).

Membrane recruitment of Kette induces F-actin formation via Wasp

Treatment of S2 cells expressing the membrane-bound Kette^{Myr} protein with *wave* dsRNA did not suppress the Kette^{Myr} induced phenotype, suggesting the Kette may induce F-actin formation independent of Wave (data not shown). Another important regulator of F-actin formation is Wasp, which has a single *Drosophila* homolog. Removal of one copy of the *wasp* gene significantly restores the bristle phenotype evoked by overexpression of Kette^{Myr} (Fig. 7D). If Kette can act via activating Wasp, similar phenotypes might be expected following disruption of either gene. This is indeed the case, and loss of zygotic and maternal Wasp function results in a *kette*-like embryonic CNS phenotype (Tal et al., 2002; Zallen et al., 2002). To test whether a reduction in the gene dose of *wasp*

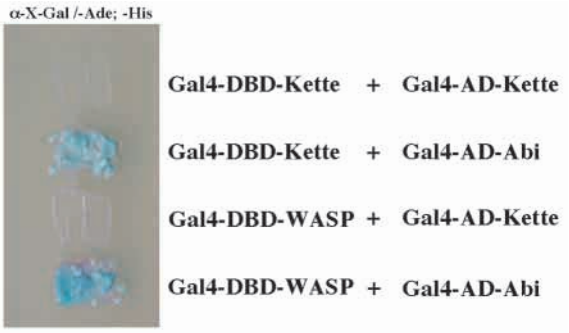


Fig. 8. Abi binds Kette and Wasp. The yeast two hybrid system was used to determine the interaction between Kette and Wasp. The indicated constructs were tested for their ability to induce three distinct selection genes (–Ade, –His and α-Gal reporters). The interactions of Kette and Abi and Wasp and Abi reconstitute functional GAL4 proteins, which enable yeast cells (AH109 strain) to grow on plates lacking adenine and histidine (Ade^{-}, His^{-}). In addition, these interactions activated α-Gal expression as demonstrated by the blue color of the colonies. Yeast cells co-transformed with the Gal4 fusions pGAD-Kette and pGBK-Kette; pGAD-Kette and pGBK-Wasp failed to grow on selection plates.

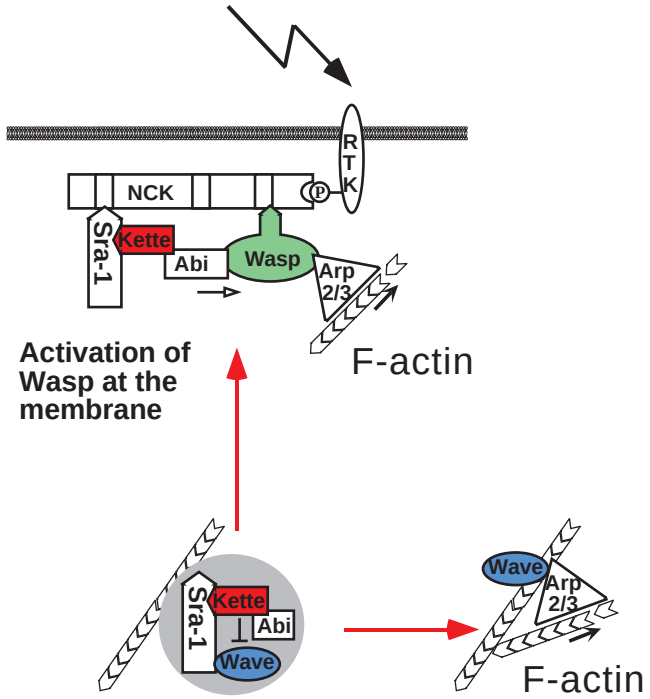


Fig. 9. Model of Kette function. Kette negatively regulates the activity of Wave in the cytosol but can activate Wasp at the membrane via Abi. Known interacting proteins are indicated. Kette also binds to F-actin, which helps to keep the Wave complex in a good position to stimulate formation of a meshed F-actin network. For further details see text.

may also affect the mutant *kette* phenotype, we generated a $kette^{C3\text{-}20}$ $wasp^{3}$ double mutant. However, the *kette wasp* phenotype appeared identical to the *kette* mutant phenotype (Fig. 6E), which is in agreement with the notion that Kette and Wasp act positively in the same pathway.

In summary, Kette appears to repress Wave and, after membrane association, is able to activate Wasp. In the cytosol Kette is found in a complex with Wave but to date no interaction between Kette and Wasp has been reported. To elucidate further how Kette may regulate Wasp activity, we determined possible protein-protein interactions between Kette and Wasp in a yeast two hybrid assay (Fig. 8). Although no direct interaction was found between Kette and Wasp, it could be demonstrated between Kette and Abi as well as between Abi and Wasp, which thus is able to link Kette and Wasp.

DISCUSSION

Regulation of growth cone and cell motility involves coordinated control of cytoskeleton dynamics (Baum and Perrimon, 2001; Mitchison and Cramer, 1996). We present biochemical and genetic evidence that the *Drosophila* Nap1 homolog, Kette, is able to modulate the activity of both Wasp and Scar/Wave and thus contribute to the regulation of F-actin dynamics. Wasp and Wave together with Cdc42 and Rac1 control different aspects of cortical actin dynamics (Hall, 1998; Takenawa and Miki, 2001). Cdc42 and Wasp are required for filopodia formation, whereas dominant-negative Wave disrupts the Rac1-dependent formation of branched network of F-actin bundles required to form lamellipodia (Fukuoka et al., 2001; Hall, 1998; Miki et al., 1998a). Wave localizes to membrane ruffles induced by activated Rac1 and Wasp accumulates in microspikes containing bundled F-actin (Miki et al., 1998b; Nakagawa et al., 2001).

Regulation of Scar/Wave activity

Recent work from the Kirschner laboratory showed that a large 500 kDa complex comprising Nap1/Kette, PIR121/Sra1, Abi, HSPC300 and Wave silences the otherwise constitutive activity of Wave in stimulating actin polymerization (Eden et al., 2002). The 500 kDa complex is stabilized by direct protein-protein interactions that have been demonstrated between Nap1/Kette and Abi (Tsuboi et al., 2002; Yamamoto et al., 2001), and Nap1/Kette and PIR121/Sra1 (also called Gex2) (Soto et al., 2002) (see Fig. 9 for a model). The exact binding partners of Wave are presently unknown.

Two modes of Wave activation have been demonstrated in vitro. On the one hand, activated Rac1 is able to bind to PIR121/Sra1 (Kobayashi et al., 1998) and can relieve Wave inhibition by dissociating the Nap1/Kette, PIR121/Sra1 and Abi sub-complex (Eden et al., 2002). On the other hand, PIR121/Sra1 is able to bind to the first SH3 domain of SH2SH3 adapter Nck (Kitamura et al., 1996; Kitamura et al., 1997), which is sufficient to activate the Wave complex (Eden et al., 2002). In vivo these two mechanisms may work at the same time to fully activate Wave.

In agreement with the work of Eden et al. (Eden et al., 2002) disruption of Kette function leads to an excess formation of cytoplasmic F-actin. However, expression of even very high levels of wild-type Kette protein do not evoke any mutant phenotype. Thus, in wild type cells the inactive Wave complexes are already formed and Kette overexpression does not result in an additional sequestering of Wave into the silencing complexes and/or an incorporation of additional

Kette into these complexes. In addition, as Kette requires Sra1 function to bind to membrane-associated adapters such as Nck, excess cytosolic Kette will not be able to reorganize subcellular Wave distribution.

Further support for the notion that Kette mediates repression of Scar/Wave activity stems from genetic analyses. Embryos lacking zygotic *kette* function display a characteristic CNS phenotype, whereas loss of zygotic Scar/Wave expression does not affect embryonic nervous system development (Zallen et al., 2002). The *kette* mutant phenotype, which is due to defects in neurite outgrowth (Hummel et al., 2000), could significantly be suppressed by reducing the dose of Scar/Wave expression. This demonstrates that in wild-type embryos, Kette acts as a negative regulator of Scar/Wave. Similar results were obtained when we reduced Kette and or Scar/Wave expression in *Drosophila* S2 cells. These experiments also revealed that Scar/Wave is required for the normal subcellular distribution of Kette, which may, however, be an indirect effect caused the disruption of the F-actin actin cytoskeleton.

Kette function at the membrane

Kette protein localizes to the plasma membrane where it accumulates in focal adhesion contacts. A prime candidate that may mediate recruitment of Kette to the membrane is the SH2 SH3 adapter protein Nck which, besides binding of the Sra1/Kette/Abi complex, also recruits numerous other proteins to focal contact sites (Li et al., 2001). Among these is Wasp, which binds to the third SH3 domain of Nck (Quilliam et al., 1996; Rivero-Lezcano et al., 1995) (Fig. 9).

The genetic interaction of *kette* and *dock* which encodes the *Drosophila* Nck homolog has recently been shown (Hummel et al., 2000). We have demonstrated that membrane recruitment of Kette is sufficient to activate actin polymerization in the cell cortex mediated by Wasp. How is this brought about? One explanation might be that recruitment of Kette to the membrane disintegrates the inhibitory Wave complex – independent of the Nck/Sra1 association. This would then lead to an excess of Wave activity and subsequently to an excess of actin polymerization. However, the genetic data clearly show that membrane bound Kette functions independent of Scar/Wave but depends on Wasp.

Genetic interaction between *kette* and *wasp*

Wasp usually adopts an auto-inhibited conformation and is activated after Cdc42, Nck binding or phosphorylation (Kim et al., 2000; Miki et al., 1998a). A structure-function analysis of the *Drosophila* Wasp demonstrated that the Cdc42-binding domain is not necessary for function, suggesting that alternative pathways, such as phosphorylation can activate Wasp (Tal et al., 2002). Kette might be a part of such an alternative pathway, as we could demonstrate a genetic interaction between *kette* and *wasp* in the regulation of actin dynamics. Regulation of Wasp by Kette is not mediated by direct protein-protein interaction, but probably involves Abi that is able to link Kette and Wasp. The Abl interactor (Abi) protein localizes to sites of actin polymerization at the tips of lamellipodia and filopodia and has been implicated in the cytoskeletal reorganization in response to growth factor stimulation (Stradal et al., 2001). As a positive regulator of the non-receptor tyrosine-kinase Abelson (Abl) Abi may bring Abl into position to phosphorylate and thus activate Wasp. Abl is known to phosphorylate many proteins regulating focal adhesion and F-actin dynamics and overexpression of activated Abl induces F-Actin formation in Cdc42-independent manner (Woodring et al., 2002). Some tyrosine kinases activate by phosphorylation of Wasp (Cory et al., 2002; Scott et al., 2002; Suetsugu et al., 2002a); however, direct phosphorylation of Wasp by Abl remains to be demonstrated.

Further support of the model (Fig. 9) that in vivo Kette activates Wasp but suppresses Wave are the phenotypic analyses of *Drosophila kette*, *wasp* and *scar/wave* mutants. Mutations in *kette* have been isolated due to defects in commissure formation in the embryonic CNS (Hummel et al., 1999). If Kette acts via activating Wasp, similar phenotypes are expected following disruption of either gene. This is indeed the case and loss of zygotic and maternal Wasp function results in a *kette*-like embryonic CNS phenotype (Hummel et al., 2000; Zallen et al., 2002).

In agreement with the proposed function of Kette in regulating both, Wasp and Wave, is its subcellular distribution. Whereas the majority of Kette is present in the cytoplasm to keep Wave in its inactive state (Eden et al., 2002) some is present leading edge of lamellipodia-like structures. However, highest amounts of Kette are present at the insertion points of large F-actin bundles where N-Wasp is also present (Nakagawa et al., 2001). Kette might be recruited to these focal adhesion sites via Sra1/Nck (Goicoechea et al., 2002) and via Wasp could enhance the formation of F-actin bundles.

How Wasp activity results in straight F-actin bundles, whereas Wave stimulates the formation of a meshed F-actin network is presently unclear (Takenawa and Miki, 2001). In the cytosol, Kette may act as a scaffold protein that keeps Wave close to F-actin and recruits additional factors to F-actin such as Profilin, which not only binds to Kette but also enhances actin nucleation (Tsuboi et al., 2002; Witke et al., 1998; Yang et al., 2000). Thus, Kette could promote the formation of a meshed F-actin network characteristic for lamellipodia. At the membrane other proteins may interact with Kette and in this respect it is interesting to note that the F-actin crosslinking protein Filamin, which plays an important role in filopodia formation, also binds to Kette (S.B., unpublished). This suggests that Kette, in addition to regulating Wasp and Wave, may also contribute to the decision whether filopodia or lamellipodia are formed.

We thank E. Schejter for sending *wave* and *scar* mutants; M. Bähler for help during the co-sedimentation experiments and reagents; M. Fröhlich and K. Krukkert for excellent technical assistance; M. Bähler, G. Edenfeld, V. Gerke and A. Püschel for many helpful discussions and comments on the manuscript; and members of the Klämbt laboratory for help throughout the project. This work was funded through a grant of the DFG to C.K. and S.B.

REFERENCES

Adler, C. E., Miyoshi-Akiyama, T., Aleman, L. M., Tanaka, M., Smith, J. M. and Mayer, B. J. (2000). Abl family kinases and Cbl cooperate with the Nck adaptor to modulate Xenopus development. *J. Biol. Chem.* **275**, 36472-36478.

Baum, B. and Perrimon, N. (2001). Spatial control of the actin cytoskeleton in Drosophila epithelial cells. *Nat. Cell Biol.* **3**, 883-890.

Baumgartner, S., Martin, D., Chiquet-Ehrismann, R., Sutton, J., Desai, A., Huang, I., Kato, K. and Hromas, R. (1995). The HEM proteins: a novel

family of tissue specific transmembrane proteins expressed from invertebrates through mammals with an essential function in oogenesis. *J. Mol. Biol.* **251**, 41-49.

Ben-Yaacov, S., le Borgne, R., Abramson, I., Schweisguth, F. and Schejter, E. D. (2001). Wasp, the Drosophila Wiskott-Aldrich syndrome gene homologue, is required for cell fate decisions mediated by Notch signaling. *J. Cell Biol.* **152**, 1-13.

Bogdan, S., Senkel, S., Esser, F., Ryffel, G. U. and Pogge v Strandmann, E. (2001). Misexpression of Xsiah-2 induces a small eye phenotype in Xenopus. *Mech. Dev.* **103**, 61-69.

Borisy, G. G. and Svitkina, T. M. (2000). Actin machinery: pushing the envelope. *Curr. Opin. Cell Biol.* **12**, 104-112.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Cory, G. O., Garg, R., Cramer, R. and Ridley, A. J. (2002). Phosphorylation of tyrosine 291 enhances the ability of WASp to stimulate actin polymerization and filopodium formation. Wiskott-Aldrich Syndrome protein. *J. Biol. Chem.* **277**, 45115-45121.

Desai, C. J., Garrity, P. A., Keshishian, H., Zipursky, S. L. and Zinn, K. (1999). The Drosophila SH2 SH3 adapter protein Dock is expressed in embryonic axons and facilitates synapse formation by the RP3 motoneuron. *Development* **126**, 1527-1535.

Eden, S., Rohatgi, R., Podtelejnikov, A. V., Mann, M. and Kirschner, M. W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* **418**, 790-793.

Fukuoka, M., Suetsugu, S., Miki, H., Fukami, K., Endo, T. and Takenawa, T. (2001). A novel neural Wiskott-Aldrich syndrome protein (N-WASP) binding protein, WISH, induces Arp2/3 complex activation independent of Cdc42. *J. Cell Biol.* **152**, 471-482.

Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T. and Zipursky, S. L. (1996). Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell* **85**, 639-650.

Goicoechea, S. M., Tu, Y., Hua, Y., Chen, K., Shen, T. L., Guan, J. L. and Wu, C. (2002). Nck-2 interacts with focal adhesion kinase and modulates cell motility. *Int. J. Biochem. Cell Biol.* **34**, 791-805.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

Higgs, H. N. and Pollard, T. D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu. Rev. Biochem.* **70**, 649-676.

Hummel, T., Schimmelpfeng, K. and Klämbt, C. (1999). Commissure formation in the embryonic CNS of *Drosophila*: I Identification of the required gene functions. *Dev. Biol.* **208**, 381-398.

Hummel, T., Leifker, K. and Klämbt, C. (2000). The Drosophila HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization. *Genes Dev.* **14**, 863-873.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.

Kim, A. S., Kakalis, L. T., Abdul-Manan, N., Liu, G. A. and Rosen, M. K. (2000). Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* **404**, 151-158.

Kitamura, T., Kitamura, Y., Yonezawa, K., Totty, N. F., Gout, I., Hara, K., Waterfield, M. D., Sakaue, M., Ogawa, W. and Kasuga, M. (1996). Molecular cloning of p125Nap1, a protein that associates with an SH3 domain of Nck. *Biochem. Biophys. Res. Commun.* **219**, 509-514.

Kitamura, Y., Kitamura, T., Sakaue, H., Maeda, T., Ueno, H., Nishio, S., Ohno, S., Osada, S., Sakaue, M., Ogawa, W. et al. (1997). Interaction of Nck associated protein 1 with activated GTP binding protein Rac. *Biochem. J.* **322**, 873-878.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H. and Klämbt, C. (1994). The Ets transcription factors encoded by the Drosophila gene pointed direct glial cell differentiation in the embryonic CNS. *Cell* **78**, 149-160.

Kobayashi, K., Kuroda, S., Fukata, M., Nakamura, T., Nagase, T., Nomura, N., Matsuura, Y., Yoshida-Kubomura, N., Iwamatsu, A. and Kaibuchi, K. (1998). p140Sra-1 (specifically Rac1-associated protein) is a novel specific target for Rac1 small GTPase. *J. Biol. Chem.* **273**, 291-295.

Li, W., Fan, J. and Woodley, D. T. (2001). Nck/Dock: an adapter between cell surface receptors and the actin cytoskeleton. *Oncogene* **20**, 6403-6417.

Li, X., Meriane, M., Triki, I., Shekarabi, M., Kennedy, T. E., Larose, L. and Lamarche-Vane, N. (2002). The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism. *J. Biol. Chem.* **277**, 37788-37797.

Machesky, L. M., Atkinson, S. J., Ampe, C., Vandekerckhove, J. and Pollard, T. D. (1994). Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. *J. Cell Biol.* **127**, 107-115.

May, R. C. (2001). The Arp2/3 complex: a central regulator of the actin cytoskeleton. *Cell Mol. Life Sci.* **58**, 1607-1626.

Miki, H., Sasaki, T., Takai, Y. and Takenawa, T. (1998a). Induction of filopodium formation by a WASP-related actin- depolymerizing protein N-WASP. *Nature* **391**, 93-96.

Miki, H., Suetsugu, S. and Takenawa, T. (1998b). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J.* **17**, 6932-6941.

Miki, H., Yamaguchi, H., Suetsugu, S. and Takenawa, T. (2000). IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. *Nature* **408**, 732-735.

Mitchison, T. J. and Cramer, L. P. (1996). Actin-based cell motility and cell locomotion. *Cell* **84**, 371-379.

Nakagawa, H., Miki, H., Ito, M., Ohashi, K., Takenawa, T. and Miyamoto, S. (2001). N-WASP, WAVE and Mena play different roles in the organization of actin cytoskeleton in lamellipodia. *J. Cell Sci.* **114**, 1555-1565.

Pantaloni, D., le Clainche, C. and Carlier, M. F. (2001). Mechanism of actin-based motility. *Science* **292**, 1502-1506.

Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M. and Weed, S. A. (2000). Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* **19**, 5606-5613.

Patel, N. H., Snow, P. and Goodman, C. S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila*. *Cell* **48**, 975-988.

Quilliam, L. A., Lambert, Q. T., Mickelson-Young, L. A., Westwick, J. K., Sparks, A. B., Kay, B. K., Jenkins, N. A., Gilbert, D. J., Copeland, N. G. and Der, C. J. (1996). Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. *J. Biol. Chem.* **271**, 28772-28776.

Rivero-Lezcano, O. M., Marcilla, A., Sameshima, J. H. and Robbins, K. C. (1995). Wiskott-Aldrich syndrome protein physically associates with Nck through Src homology 3 domains. *Mol. Cell. Biol.* **15**, 5725-5731.

Robinson, R. C., Turbedsky, K., Kaiser, D. A., Marchand, J. B., Higgs, H. N., Choe, S. and Pollard, T. D. (2001). Crystal structure of Arp2/3 complex. *Science* **294**, 1679-1684.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M. W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42- dependent signals to actin assembly. *Cell* **97**, 221-231.

Schafer, D. A. and Cooper, J. A. (1995). Control of actin assembly at filament ends. *Annu. Rev. Cell Dev. Biol.* **11**, 497-518.

Scott, M. P., Zappacosta, F., Kim, E. Y., Annan, R. S. and Miller, W. T. (2002). Identification of novel SH3 domain ligands for the Src family kinase Hck. Wiskott-Aldrich syndrome protein (WASP), WASP-interacting protein (WIP), and ELMO1. *J. Biol. Chem.* **277**, 28238-28246.

Simon, M. A., Drees, B., Kornberg, T. and Bishop, J. M. (1985). The nucleotide sequence and the tissue-specific expression of Drosophila c-src. *Cell* **42**, 831-840.

Soto, M. C., Qadota, H., Kasuya, K., Inoue, M., Tsuboi, D., Mello, C. C. and Kaibuchi, K. (2002). The GEX-2 and GEX-3 proteins are required for tissue morphogenesis and cell migrations in C. elegans. *Genes Dev.* **16**, 620-632.

Spudich, J. A. and Watt, S. (1971). The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**, 4866-4871.

Stradal, T., Courtney, K. D., Rottner, K., Hahne, P., Small, J. V. and Pendergast, A. M. (2001). The Abl interactor proteins localize to sites of actin polymerization at the tips of lamellipodia and filopodia. *Curr. Biol.* **11**, 891-895.

Suetsugu, S., Hattori, M., Miki, H., Tezuka, T., Yamamoto, T., Mikoshiba, K. and Takenawa, T. (2002a). Sustained activation of N-WASP through phosphorylation is essential for neurite extension. *Dev. Cell* **3**, 645-658.

Suetsugu, S., Miki, H. and Takenawa, T. (2002b). Spatial and temporal regulation of actin polymerization for cytoskeleton formation through Arp2/3 complex and WASP/WAVE proteins. *Cell Motil. Cytoskel.* **51**, 113-122.

Takenawa, T. and Miki, H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J. Cell Sci.* **114**, 1801-1809.

Tal, T., Vaizel-Ohayon, D. and Schejter, E. D. (2002). Conserved interactions with cytoskeletal but not signaling elements are an essential aspect of Drosophila WASp function. *Dev. Biol.* **243**, 260-271.

Tsuboi, D., Qadota, H., Kasuya, K., Amano, M. and Kaibuchi, K. (2002). Isolation of the interacting molecules with GEX-3 by a novel functional screening. *Biochem. Biophys. Res. Commun.* **292**, 697-701.

Wagner, C. R., Mahowald, A. P. and Miller, K. G. (2002). One of the two cytoplasmic actin isoforms in Drosophila is essential. *Proc. Natl. Acad. Sci. USA* **99**, 8037-8042.

Welch, M. D., DePace, A. H., Verma, S., Iwamatsu, A. and Mitchison, T. J. (1997). The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. *J. Cell Biol.* **138**, 375-384.

Witke, W., Podtelejnikov, A. V., di Nardo, A., Sutherland, J. D., Gurniak, C. B., Dotti, C. and Mann, M. (1998). In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly. *EMBO J.* **17**, 967-976.

Woodring, P. J., Litwack, E. D., O'Leary, D. D., Lucero, G. R., Wang, J. Y. and Hunter, T. (2002). Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. *J. Cell Biol.* **156**, 879-892.

Yamamoto, A., Suzuki, T. and Sakaki, Y. (2001). Isolation of hNap1BP which interacts with human Nap1 (NCKAP1) whose expression is down-regulated in Alzheimer's disease. *Gene* **271**, 159-169.

Yang, C., Huang, M., DeBiasio, J., Pring, M., Joyce, M., Miki, H., Takenawa, T. and Zigmond, S. H. (2000). Profilin enhances Cdc42-induced nucleation of actin polymerization. *J. Cell Biol.* **150**, 1001-1012.

Zallen, J. A., Cohen, Y., Hudson, A. M., Cooley, L., Wieschaus, E. and Schejter, E. D. (2002). SCAR is a primary regulator of Arp2/3-dependent morphological events in Drosophila. *J. Cell Biol.* **156**, 689-701.

Zhang, C. X. and Hsieh, T. (2000). Preparation of membrane proteins from Drosophila embryos. In Drosophila. *A Laboratory Manual* (ed. W. Sullivan, M. Ashburner and S. Hawley), pp. 564-569. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.