Sra-1 interacts with Kette and Wasp and is required for neuronal and bristle development in *Drosophila*

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Summary

Regulation of growth cone and cell motility involves the coordinated control of F-actin dynamics. An important regulator of F-actin formation is the Arp2/3 complex, which in turn is activated by Wasp and Wave. A complex comprising Kette/Nap1, Sra-1/Pir121/CYFIP, Abi and HSPC300 modulates the activity of Wave and Wasp. We present the characterization of *Drosophila* Sra-1 (specifically Rac1-associated protein 1). *sra-1* and *kette* are spatially and temporally co-expressed, and both encoded proteins interact in vivo. During late embryonic and larval development, the Sra-1 protein is found in the neuropile. Outgrowing photoreceptor neurons express high levels of Sra-1 also in growth cones. Expression of double stranded *sra-1* RNA in photoreceptor neurons leads to a stalling of axonal growth. Following knockdown of *sra-1* function in motoneurons, we noted abnormal neuromuscular junctions similar to what we determined for hypomorphic *kette* mutations. Similar mutant phenotypes were induced after expression of membrane-bound Sra-1 that lacks the Kette-binding domain, suggesting that *sra-1* function is mediated through *kette*. Furthermore, we could show that both proteins stabilize each other and directly control the regulation of the F-actin cytoskeleton in a Wasp-dependent manner.

Supplemental data available online

Key words: F-actin, *Drosophila*, NAP1/Kette, Sra-1/CYFIP, Wasp

Introduction

The cytoskeleton plays a central role in cell morphology and motility. In particular the F-actin cytoskeleton of most eukaryotic cells is dynamically reorganized, providing force for shape changes and movements (Borisy and Svitkina, 2000; Pantaloni et al., 2001). This is particularly evident during nervous system development, when growth cones must advance and synaptic plasticity has to be regulated. 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 (Pollard and Borisy, 2003). Actin dynamics is controlled by a small set of proteins. The Arp2/3 complex has been shown to nucleate de novo actin polymerization (Machesky et al., 1994). Arp2/3 is normally repressed, but can be activated by the members of the Wiskott family Wasp and Wave.

The Wasp proteins are auto-inhibited, whereas the Wave proteins are trans-inhibited. Both usually require small G proteins of the Rho family for activation (Miki and Takenawa, 2003). In the case of Wasp, activated, GTP-bound Cdc42 binds 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). However, a structure-function analysis of the *Drosophila* Wasp has demonstrated that the Cdc42-binding domain is not strictly necessary for function, suggesting that alternative pathways, such as phosphorylation can activate WASP (Tal et al., 2002). Indeed, some tyrosine kinases have been shown to activate Wasp by phosphorylation (Cory et al., 2002; Scott et al., 2002; Suetsugu et al., 2002).

In contrast to Wasp, Wave is not auto-inhibited. It is kept in an inactive state through association with a protein complex comprising Kette/Nap1, Sra1 (specifically Rac associated 1, Sra-1; also called CYFIP/p140Sra-1, from here on called Sra-1 according to FlyBase) and the Abelson-interactor protein (Abi) (Eden et al., 2002). Upon dissociation or conformational changes of this complex, Wave is assumed to be active (Eden et al., 2002; Steffen et al., 2004). Thus, Kette or Sra-1 should antagonize Wave function. This is supported by genetic studies in *Dictyostelium* and *Drosophila* (Blagg et al., 2003; Bogdan and Klämbt, 2003). Cell culture experiments show that Wave is degraded in a Ubiquitin-dependent manner following disruption of the Sra-1/Kette complex (Kunda et al., 2003; Rogers et al., 2003). These latter findings are likely to reflect the fast inactivation of Wave once activated. In vitro, activation of Wave can be mediated by Rac1 or SH3 domains, which presumably bind to Sra-1 (Eden et al., 2002; Kobayashi et al., 1998; Miki et al., 2000).

The Sra-1/Kette protein complex is not only required to negatively regulate the activity of Wave but is also able to activate Wasp function at the membrane (Bogdan and Klämbt, 2003). The interaction of Kette and Wasp is not direct but is likely to be mediated by the Abi, which can bind to both Kette and Wasp (Bogdan and Klämbt, 2003). Interestingly, the Nck adapter protein is also able to bind to Wasp via its third SH3 domain (Rivero-Lezcano et al., 1995). Thus, Sra-1, which can

bind to the first SH3 domain of Nck is a good candidate to locate the Sra-1/Kette complex to the membrane close to Wasp (Kitamura et al., 1996; Kitamura et al., 1997; Kobayashi et al., 1998).

To test whether the mutant phenotypes of *sra-1* and *kette* are alike as predicted, and whether Sra-1 indeed acts through Kette to regulate actin dynamics, we conducted a functional characterization of Sra-1 during *Drosophila* development. Sra-1 and Kette are both required for axonal growth and perform common functions during formation and maturation of neuromuscular junctions (NMJ). Analysis of temporal and spatial distribution of the Sra-1 protein shows a prominent co-expression with Kette. Both proteins are maternally expressed and later in development become concentrated in the developing nervous system (CNS). Sra-1 is highly expressed in growth cones and neuromuscular synapses. Direct interaction of Sra-1 and Kette depends on a short C-terminal domain of the Sra-1 protein. Expression of a Sra-1 variant lacking the C-terminal domain leads to a dominant-negative phenotype that can be suppressed by expression of an activated Kette protein. In tissue culture cells as well as in vivo Sra-1 function is required for F-actin organization. Further genetic analyses demonstrate that Sra-1 function at the membrane depends on the presence of Wasp.

Materials and methods

Genetics

All crosses were performed at 25°C unless otherwise indicated. The following strains were used: $wasp^{1}$/TM6 $wasp^{3}$/TM; $kette^{J4\text{-}48}$/TM6, $kette^{\Delta 2\text{-}6}$/TM6 and $kette^{J1\text{-}70}$/TM6 (Hummel et al., 2000). To determine the gain- and loss-of-function phenotype of $Kette^{Myr}$, Sra^{Myr}, $SraΔC^{Myr}$ and Sra-RNAi, we used the GAL4 system (Brand and Perrimon, 1993). Transgenic flies carrying the following constructs were generated following standard procedures: UAS-$kette^{Myc}$; UAS-sra^{Myc}; UAS-*sra*-$ΔC^{Myc}$ and UAS-*sraRNAi*. The $Kette^{Myr}$, Sra^{Myr} and $SraΔC^{Myr}$ constructs were made by fusing DNA encoding the first 88 amino acids from *Drosophila Src1* (Simon et al., 1985) to the first codon of Kette, Sra-full-length and Sra^{1151aa}. To generate the Sra-RNAi construct, the *sra* DNA (coding sequence +1 to +300, containing no repetitive sequence motif) was cloned as an inverted repeat into pWIZ (Lee and Carthew, 2003). Several independent transgenic lines were analyzed in each case.

Antibody production

The rabbit anti-Sra antibody (Sra900) was generated against the first N-terminal 300 amino acids of Sra fused with a His_6-tag (Qiagen). The His_6-Sra fusion protein was expressed in *E. coli* and purified with Ni-NTA resin (Qiagen) under native conditions. Rabbits were immunized with purified proteins by Davids Biotechnologie (Germany).

In situ hybridization, immunolabeling and western blot analysis

In situ hybridization and immunohistochemistry was performed as described (Hummel et al., 1997; Tautz and Pfeifle, 1989). *Sra*-specific riboprobes were generated from EST clones LD47929 and LD19991. Larval dissections and antibody staining were carried out as previously described (Schuster et al., 1996). Antibodies were used as follows: affinity-purified anti-Sra polyclonal antibody, 1:100; mAB nc46 (Reichmuth et al., 1995), 1:10; mAB BP102, 1:100 (Developmental Studies Hybridoma Bank). For protein extracts, staged embryos were mashed with a pestle in lysis buffer (1×PBS, 1% NP40, 1 mM DTT and 1 mM PMSF). Lysates were centrifuged 10 minutes at 10,000 ***g*** to yield the cytoplasmic supernatant. The amount of total protein was determined by a Bradford assay (BioRad). Equivalent amounts of protein extract were separated by SDS-PAGE, and analyzed by western blot as described previously (Bogdan et al., 2001).

Two-hybrid assay

To test the interaction between Sra-1 and Kette, we inserted the *kette* ORF in frame with the GAL4 DNA-binding domain into the GBK-T7 vector (Clontech) and the Sra-1 full-length and C-terminal deletion of Sra-1 in frame with the GAL4 activation domain into the GAD-T7 vector (Clontech).

Cell culture and immunofluorescence microscopy

Drosophila S2R+ (Yanagawa et al., 1998) 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^2 T-flasks (Sarstedt) at 25°C. For immunofluorescence microscopy, $2×10^5$ cells were plated on glass cover slips (pretreated with or without fibronectin) in 24-well plates, cultured for 24 hours and fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, stained with *Drosophila* anti-Kette antibody 97/82 (1:2000 dilution) or purified anti-Sra antibody (Sra900) 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. The samples were mounted in a 25% (w/v) Mowiol (Sigma) solution containing DABCO (Sigma) and visualized on a Leica LSM.

Cell transfection and co-immunoprecipitation

For co-immunoprecipitation of Myc-tagged Kette protein, *Drosophila* Schneider S2 cells (10^7) were co-transfected with UAS-*kette-7xmyc* (2 μg) and Act5C-GAL4 DNA (2 μg) by *Fugene* (Roche) as described previously (Bogdan et al., 2001). Transfected cells were harvested and lysed in ice-cold lysis buffer (1×PBS, 0.5% NP40, 1 mM DTT and 1 mM PMSF). Lysates were centrifuged 10 minutes at 10,000 ***g*** to yield the cytoplasmatic supernatant. Total cell lysate was subjected to immunoprecipitation with monoclonal anti-Myc (9E10) immobilized by protein G beads. Immunoprecipitates were separated by SDS-PAGE, and proteins were analyzed by western blot as described previously (Bogdan et al., 2001) using polyclonal antiserum to Sra (Sra900).

dsRNA production and RNAi treatment

sra-1 ORF (300 bp) and *kette* ORF (570 bp) were inserted into the vector pLITMUS-28i (BioLabs) flanked by T7 promoters. After linearization, dsRNAs were generated using a HiScribe RNAi Transcription Kit (BioLabs) as described previously (Bogdan et al., 2001). dsRNA-treated cells were incubated for additional 2-3 days and analyzed by immunofluorescence and western blot analysis.

Results

sra-1 and *kette* are co-expressed and interact in vivo

kette and *sra-1* are initially broadly expressed during embryogenesis (Hummel et al., 2000; Schenck et al., 2003) (see Fig. S1 at http://dev.biologists.org/supplemental). At the end of embryogenesis, RNA expression of both genes becomes restricted to the CNS (Fig. 1A). To further analyze the expression of Sra-1 we generated anti-Sra-1 antibodies (see Materials and methods for details). Western blot analyses show expression of Kette and Sra-1 throughout development (Fig. 1B). Antisera against the two proteins each recognize a single

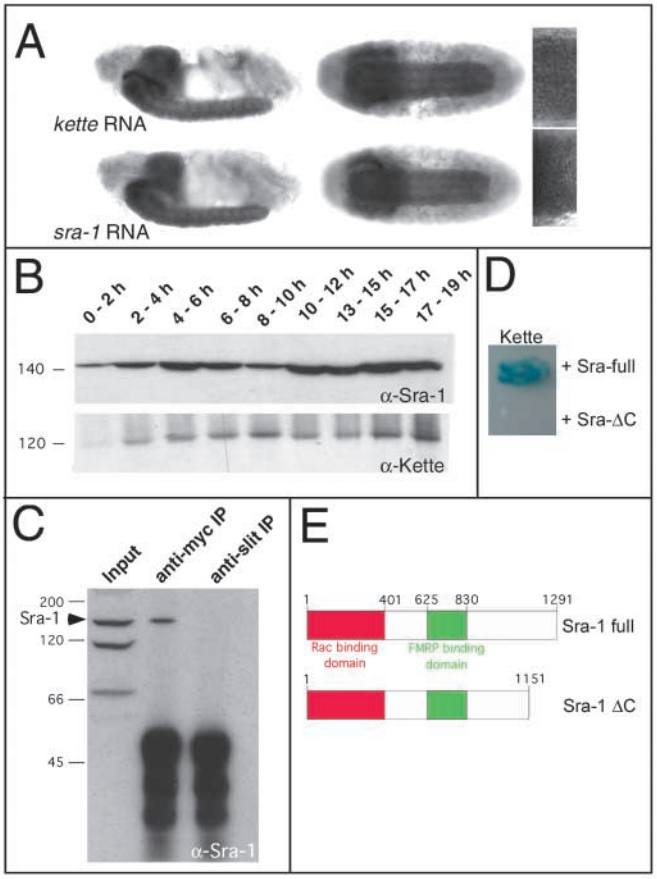

Fig. 1. Sra-1 binds Kette through its C terminus. (A) Whole-mount stage 16 embryos stained for *kette* or *sra-1* RNA expression as indicated, (Left) Lateral views; (middle) ventral views; (right) higher magnifications of the ventral nerve cord showing ubiquitous expression of both genes. (B) Western blot analyses of Kette (120 kDa) and Sra-1 (140 kDa) expression. Both proteins are expressed throughout embryogenesis and appear to be provided maternally. (C) Co-immunoprecipitation of Kette and Sra-1. Total cell lysate of S2-cells expressing both Myc-tagged Kette and Sra-1 was subjected to immunoprecipitation with monoclonal anti-Myc (9E10) or anti-Slit (E555.6D). Sra-1 is detected only in the anti-Myc precipitate, indicating binding of Kette and Sra-1. (D) To test the interaction between Sra-1 and Kette, we turned to a yeast two hybrid assay. Interaction between Sra-1 and Kette results in growth of the yeast cells and a blue color. Interaction is seen only when the C terminus is intact (E).

band, which is present in cell extracts from first cleavage stage embryos to hatching (Fig. 1B).

As both proteins are co-expressed during development and their vertebrate homologues are found in the same protein complex, we asked whether the two proteins could physically interact. First, we transfected *Drosophila* S2 tissue culture cells with a vector encoding a Myc-tagged Kette protein and cell lysates were subsequently subjected to immunoprecipitation. Sra-1 protein at the expected size of 140 kDa was only detected in these precipitates, when we precipitated Kette using anti-Myc antibodies but not when we used anti-Slit antibodies as a control (Fig. 1C). This co-immunoprecipitation indicates a direct binding of Kette and Sra-1. To further verify this assumption and to map the domain on the Sra-1 protein relevant for this interaction, we used the yeast two-hybrid system. We found that full-length Sra-1 protein efficiently binds to full-length Kette protein (Fig. 1D). A small deletion of the last 100 C-terminal amino acids of the Sra-1 protein was sufficient to abrogate the interaction between Sra-1 and Kette, suggesting that the C-terminus of the Sra-1 protein is essential for the interaction with Kette. In addition to Kette, the 140 kDa protein Sra-1 has been reported to bind to Nck, Rac1 and FMRP, indicating that Sra-1 acts as a scaffolding protein linking a larger number of proteins (Fig. 1E) (Kobayashi et al., 1998; Schenck et al., 2003).

Sra-1 expression during neuronal development

Purified anti-Sra-1 antisera allow detection of the endogenous Sra-1 protein. The specificity of these antisera was confirmed by Sra-1 RNA interference (RNAi), which removed Sra-1 staining (see Figs 3, 5). In agreement with high levels of RNA expression in the CNS, we noted highest level of Sra-1 protein in the embryonic CNS neuropile, which consists of axons, dendrites and synapses (Fig. 2A). Similarly, Sra-1 is expressed in the optic lobe neuropile of third instar larvae (Fig. 2B). Here, high levels of Sra-1 protein expression can be noted in a zone between the developing lamina and medulla (Fig. 2B, asterisk). EM-analyses have previously showed that this region consists of growth cones of the developing photoreceptor neurons which also express the 24B10 epitope (Garrity et al., 1999; Garrity et al., 1996; Van Vactor et al., 1988) (Fig. 2B). In addition, we noted some Sra-1 protein in eye imaginal discs cells (Fig. 2C).

sra-1 is required for neuronal development

The expression of Sra-1 in growth cones suggests that it may be required for axonal pathfinding or targeting. To test this assumption, we generated transgenes expressing a double stranded *sra-1* RNA under the control of the UAS sequences to perform in vivo RNAi experiments (Lee and Carthew, 2003). In wild-type eye discs, photoreceptor neurons express Sra-1 and the 24B10 marker as they navigate through the optic stalk to the lamina and medulla in the brain (Fig. 3A). After expression of the *sra-1* RNAi construct in the developing eye disc using the *eyeless*-GAL4 driver, Sra-1 protein expression is removed in the eye disc but not in the brain, demonstrating the specificity of the antisera (Fig. 3B). Upon reduction of Sra-1 expression, photoreceptor neurons exhibit pronounced axonal defects. The majority of the axons appear to be able to enter the brain, but then axons fail to grow towards their correct targets (Fig. 3B). In addition, fewer photoreceptor cells develop and the compound eyes appear slightly smaller and have a rough appearance compared to the wild type (Fig. 3C,D). These phenotypes are highly penetrant and can be observed in every fly expressing *sra-1* RNAi (n>50).

sra-1 function is not restricted to axonal pathfinding. Recently, defects in synaptic architecture were found in *sra-1* mutants (Schenck et al., 2003). Similarly, inactivation of *sra-1* by RNAi specifically in neuronal cells using *elav*-GAL4 led to synaptic boutons with supernumerary buds (2.0-fold increase compared with wild type, terminal boutons of 60 neuromuscular junctions on muscle 4 of 15 larvae were counted) and a pronounced bulged 3D structure (Fig. 4B). The cell type specific *sra-1* knockdown demonstrates a cell-

autonomous requirement for *sra-1* function in the developing presynaptic neuron.

We then asked whether the interacting protein Kette is similarly required for synaptic development. Certain hypomorphic allele combinations allow development of third instar larvae (Hummel et al., 2000). *kette*$^{J1\text{-}70}$/*kette*$^{\Delta 2\text{-}6}$ gives rise to larvae with smaller neuromuscular junctions carrying bulged synaptic boutons of irregular shape resembling the *sra-1* phenotype (Fig. 4C; 2.1-fold increase of bud number of terminal boutons on muscle 4, n=60). As Sra-1 was shown to bind Kette via its C terminus (Fig. 1), we assumed that expression of a Sra-1 protein lacking this domain (Sra-1ΔC) may interfere with synaptic development. As wild-type Kette appears to function at the membrane, we generated a membrane tethered Sra-1ΔC protein variant (Sra-1ΔC^{Myr}).

Indeed, when we expressed such a construct in all neurons using the elav-GAL4 driver, we noted a synaptic phenotype similar to the one caused by reduction of *sra-1* function by RNAi (Fig. 4D; 2.3-fold increase of bud number of terminal boutons on muscle 4, n=60).

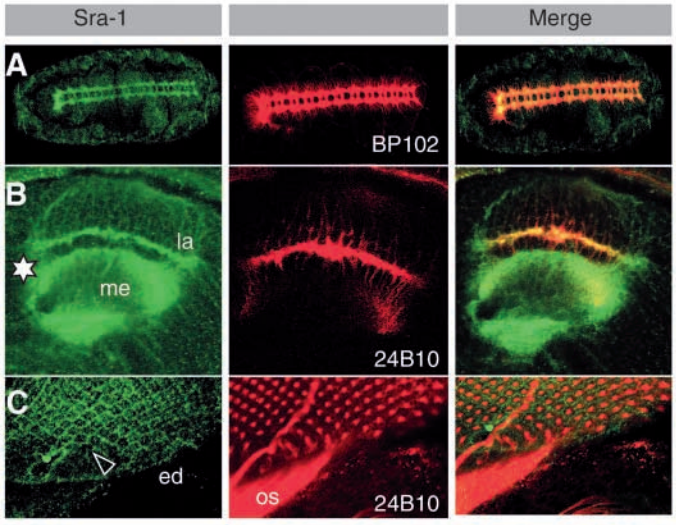

Fig. 2. Neuronal expression of Sra-1. Confocal images of mount preparations for Sra-1 expression (green) and axonal markers 24B10 or BP102 (red). The merge channel is shown on the right. (A) During embryogenesis Sra-1 (green) accumulates in the neuropile of the central nervous system, which also expresses the BP102 epitope. (B) In the optic ganglia of third instar larval brains Sra-1 protein expression can be detected in the medulla (md) and photoreceptor axons that traverse the lamina (la). An intense label can be seen in the termination zone of the photoreceptor axons R1-R6 in the lamina (star). The growth cones of these axons also express high levels of the 24B10 antigen (red). (C) Cell bodies of the photoreceptor neurons also express Sra-1, albeit at lower levels (arrowhead). ed, eye imaginal disc; os, optic stalk.

Kette and Sra-1 stabilities are mutually dependent

A number of reports have recently shown that Sra-1 and Kette stabilize the Arp2/3 activator Wave (Blagg et al., 2003; Kunda et al., 2003; Rogers et al., 2003). Degradation of Wave following disruption of the regulatory complex may be an efficient way to terminate Wave activity. If all protein members of the complex are required to keep Wave inactive, one might expect that removal of Kette or Sra-1 should also lead to a downregulation of the remaining proteins of the complex. We have tested this assumption by following Kette or Sra-1 protein expression in S2 cells in which *kette* or *sra-1* function was knocked down by RNAi. As previously shown, gene silencing of *kette* and *sra-1* in *Drosophila* S2 by RNAi cell line induced identical morphological phenotypes (Blagg et al., 2003; Bogdan and Klämbt, 2003; Kunda et al., 2003; Rogers et al., 2003). In comparison with wild-type cells, dsRNA-treated cells assume a starfish-like morphology with multiple filopodia like extensions and a prominent accumulation of cytosolic F-actin (Fig. 5, arrowheads). Immunofluorescence and western blot

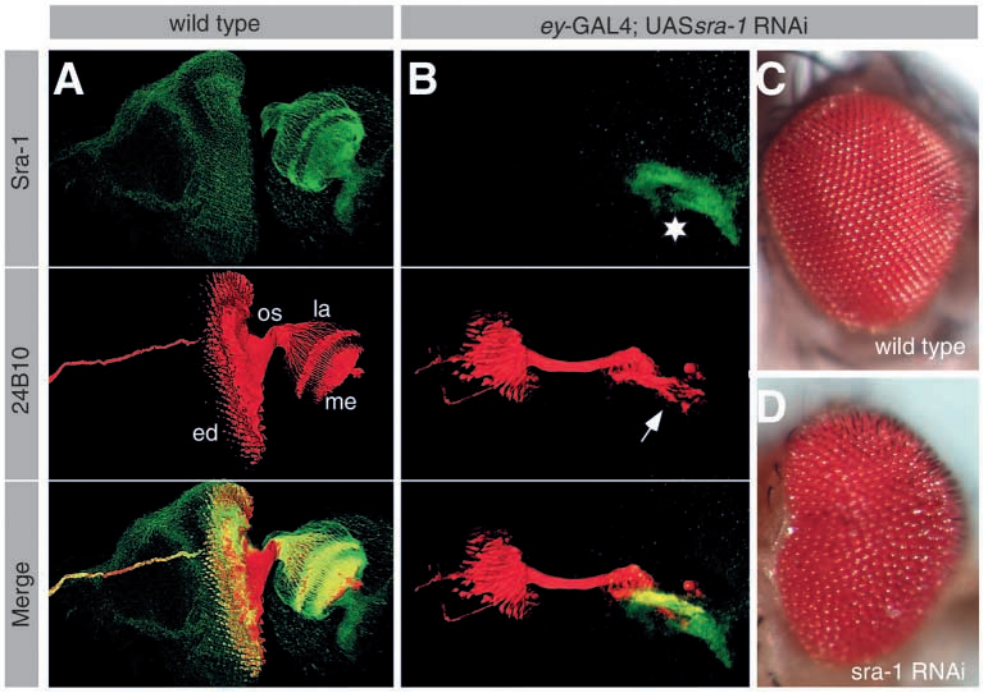

Fig. 3. *sra-1* is required for axonal growth. Confocal images of mount preparations of third instar eye discs (ed) for Sra-1 expression (green) and the axonal marker 24B10 (red). The merge channel is shown in the bottom row. (A) In wild type, photoreceptor neurons express both Sra-1 and 24B10, and project their axons through the optic stalk (os) to the lamina (la) or medulla (me). (B) Following expression of sra-1 dsRNA using an *eyeless*-GAL4 driver, Sra-1 protein expression was removed from the eye disc but not the brain (star). Concomitantly, we observed severe axonal targeting defects. Most axons appear to be able to enter the brain, but fail to grow towards their correct targets (arrow). (C,D) Adult eyes of (C) a wild-type fly and (D) a transgenic fly expressing *sra-1* RNAi in the *eyeless* pattern.

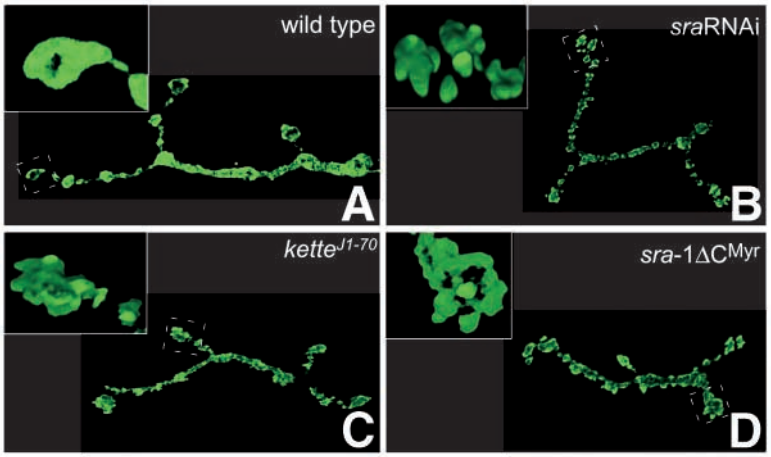

Fig. 4. *kette* and *sra-1* affect synaptic development. Neuromuscular junctions (NMJ) of muscle 4 taken from third instar larvae are shown. Boutons are labeled by Nc46 expression. The inlay shows a higher magnification of a terminal bouton highlighted by the boxed area. (A) Wild-type NMJ. Note the smooth morphology of the terminal boutons. Almost no extra branches or buds can be detected. (B) After *sra-1* RNA interference, an increased budding of terminal boutons was noted (*elav*Gal4×UAS*sra-1*^{RNAi}). (C) Third instar larvae carrying the weak hypomorphic allele combination *kette*$^{J1\text{-}70}$/*kette*$^{2\Delta6}$. There is an increase in budding tendency, which is particularly evident at the terminal boutons. (D) A similar phenotype was observed following neuronal expression of the *sra-1ΔC*^{Myr} deletion construct (*elav*Gal4×UAS*sra-1ΔC*^{Myr}).

analyses demonstrate that *kette* RNAi efficiently eliminates Kette protein expression (Fig. 5B,D). Interestingly, *kette* RNAi also reduces Sra-1 expression (Fig. 5). Conversely, when we reduced *sra-1* function by RNAi, we blocked both Sra-1 and Kette protein expression (Fig. 5C,D). These data suggest that Kette and Sra-1 are closely linked and stabilize each other.

Sra-1 is required for bristle formation

It has recently been shown that *kette* is required for F-actin formation (Bogdan and Klämbt, 2003; Hummel et al., 2000; Kunda et al., 2003; Rogers et al., 2003). As the dynamics of the F-actin cytoskeleton is difficult to analyze in *Drosophila* neurons, we turned to the analysis of bristle development which crucially depends on F-actin dynamics (Jacinto and Baum, 2003). Hypomorphic *kette* mutants (*kette*$^{J1\text{-}70}$/*kette*$^{\Delta2\text{-}6}$) are characterized by typical bent bristles (Fig. 6A, arrow; about 15% of the heads have one or more bent bristles, *n*=250). Given the general colocalization of Kette and Sra-1 and the mutual stabilization of the two proteins, we anticipated that *sra-1* is also required for bristle development. Following expression of *sra-1* dsRNA in the *scabrous* pattern, and formation of microchaete and macrochaete, we observed bent bristles similar to those in *kette* mutants (Fig. 6B, arrow; about 10% of the heads show one or more bent bristles, *n*=170). When we increased the number of *sra-1* RNAi transgenes, the penetrance of the bent bristle phenotype increased (about 30% of the heads show one or more bent bristles, *n*=100). In addition, we observed a loss of some bristles in flies expressing high levels of sra-1 RNAi (in 5% of the heads, *n*=100). A similar increase in the phenotypic penetrance was observed when we lowered the dose of *kette* in flies expressing one copy of the *sra-1* RNAi construct.

Expression of the Sra-1ΔC^{Myr} protein lacking the Kette interaction domain, resulted in a similar bristle phenotype. When one copy of the transgene was expressed, all heads lacked bristles. The few remaining bristles are frequently bended (Fig. 6C). When we increased the dose of the *sra-1ΔC*^{Myr} transgene, almost no bristles develop (data not shown).

Sra-1 affects F-actin formation

To more directly test a possible role of *sra-1* on F-actin formation, we performed phalloidin staining of bristles during pupal development. In wild-type animals, bristles are prefigured by pointed F-actin bundles (Fig. 7A). Cortical F-actin is found in a sharp zone close to the cell membrane. When we expressed *sra-1* dsRNA, pointed F-actin bundles still form, but they show bent morphology (as do the later bristles) (Fig. 7B). When we expressed the Sra-1ΔC^{Myr} construct, no F-actin bundles were detected at the site where bristles are expected to develop, corresponding to the loss of bristles observed in adult stages.

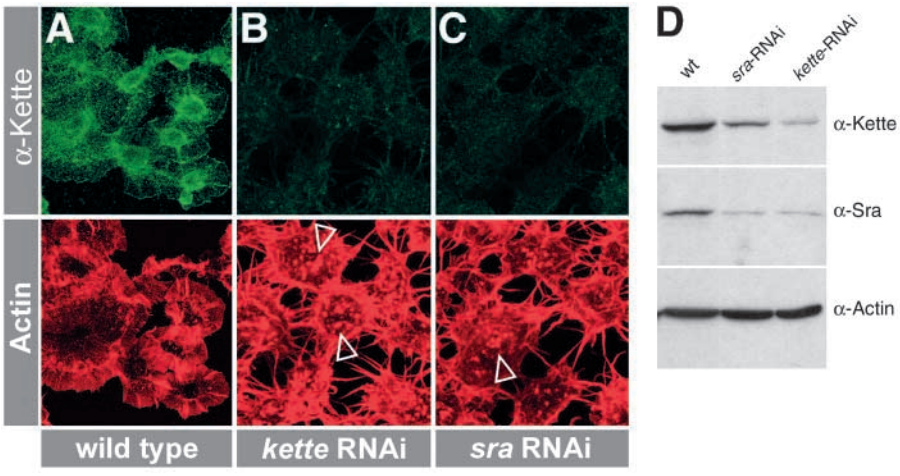

Fig. 5. Kette and Sra-1 stabilize each other. (A-C) Top row, expression of Kette (green) in S2R+ cells; bottom row, F-actin was detected using phalloidin (red). (A) Wild-type S2R+ cells grown on an adhesive substrate. (B) Kette expression in S2R+ cells can be suppressed by treatment with double-stranded *kette* RNA (RNAi). Retraction fibers and clumps of F-actin are found within the cells (arrowheads). (C) When *sra-1* function is inhibited by *sra-1* RNAi, Kette protein expression cannot be detected after 2 days of RNAi treatment. In addition, a F-actin phenotype develops (compare B with C). (D) Western blot analysis showing mutual protein stabilization of Kette and Sra-1. *sra-1* RNAi affects Sra-1 and Kette expression and, conversely, *kette* RNAi efficiently reduces Kette and Sra-1 protein expression.

We have recently shown that a membrane-tethered form of Kette behaves like an activated protein variant that efficiently reorganizes the formation of F-actin via modulating the activity of Wasp. When expressed in the developing notum of a fly, this results in the formation of branched or brushed bristles (Bogdan and Klämbt, 2003). If both Kette and Sra-1 act in the same complex, expression of a membrane-bound Sra-1 is expected to act like the membrane-bound Kette protein. Indeed, expression of a myristylated full-length Sra-1 protein (Sra-1^{Myr}) in the *scabrous* pattern induced the formation of brushed F-actin bundles (Fig. 7D, arrow). Furthermore the cortical F-actin appeared fuzzier and many irregular F-actin fibers can be recognized (Fig. 7D, arrowhead).

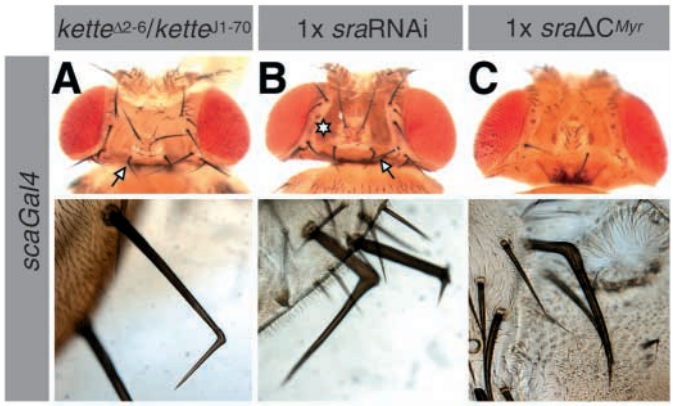

Fig. 6. *kette* and *sra-1* function during bristle development. (A-C) Top row, dorsal view of *Drosophila* heads; bottom row, higher magnification showing the morphology of macrochaetes. (A) Hypomorphic *kette* mutant flies (*kette$^{J1\text{-}70}$/kette$^{\Delta 2\text{-}6}$*) are characterized by bent bristles (arrow). (B) Following expression of *sra-1* dsRNA in the *scabrous* pattern, similar bent bristles are formed (arrow). Some bristles are missing (star). This phenotypic trait increases following expression of higher levels of Sra-1 RNAi. (C) After expression of a membrane-tethered Sra-1 protein variant lacking the Kette interaction domain (Sra-1ΔC^{Myr}), frequent loss of bristles as well as bent bristles is observed.

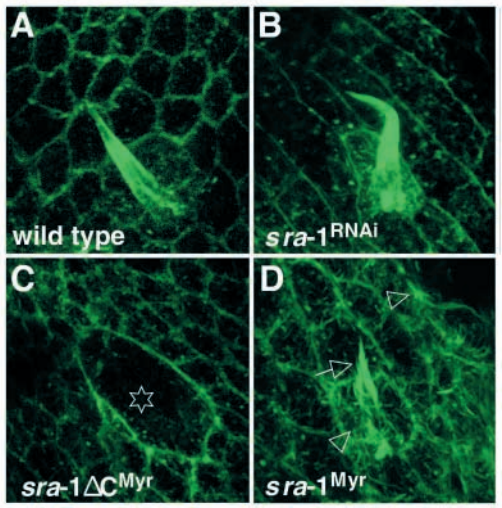

Fig. 7. Sra-1 affects F-actin formation. During pupal development, bristle morphology is prefigured by an apical F-actin extension, which can be visualized by phalloidin staining. All pupae were dissected 36 hours after puparium formation. (A) Wild type. (B) After expression of *sra-1*, dsRNA expression (one copy) using sca-GAL4 bent F-actin bundles, which are never seen in wild type, can be detected. (C) After expression of one copy of the sra-1ΔCMyr construct, no F-actin forms at the site where bristles are expected to develop (star). (D) Following expression of full-length membrane tethered Sra-1 protein using the sca-GAL4 driver, F-actin formation is initiated in a broader region of the apical cell surface (arrow). In addition, the F-actin at the cell boundary appears to have a 'fuzzier' organization (arrowheads).

Genetic interaction between *sra-1*, *kette* and *wasp*

The F-actin phenotype seen following expression of the Sra-1^{Myr} protein resulted in the formation of split bristles on the notum of the adult fly (Fig. 8B, about 10% of the microchaete, see Fig. 8I). Expression of a membrane-tethered Kette protein resulted in a similar, Wasp-dependent, split bristle phenotype (Fig. 8C,I) (Bogdan and Klämbt, 2003). Animals expressing both membrane-tethered proteins display a synergistic effect (Fig. 8C,I). To test whether the induction of the Sra-1^{Myr} induced phenotype also depends on the function of both *kette* and *wasp* we expressed Sra-1^{Myr} in heterozygous *kette* and *wasp* flies. Whereas the reduction in the gene dose of *kette* did not significantly modify the Sra-1^{Myr} induced phenotype, heterozygous loss of *wasp* function clearly suppressed the branched bristle phenotype evoked by Sra-1^{Myr} expression (Fig. 8E,F,I). This indicates that either Sra-1 does not require Kette to activate Wasp or the reduction in Kette expression level is not sufficient to detect phenotypic changes.

Expression of the dominant-negative Sra-1ΔC^{Myr} protein variant did not induce a branched bristle phenotype but led to a loss of bristles (Fig. 6C, Fig. 8G,I). This bristle phenotype could be suppressed by the expression of activated Kette^{Myr} (Fig. 8H,I). Furthermore, flies expressing both Sra-1ΔC^{Myr} and Kette^{Myr} show similarly split bristles as do flies expressing only the Kette^{Myr} protein. Thus, activated Kette acts downstream or independent of Sra-1 to regulate the activity of the F-actin regulator Wasp.

Discussion

Axonal pathfinding and synaptic growth both required well regulated F-Actin dynamics. We have analyzed the role of Sra-1, which (together with Kette) constitutes an important regulator of Wasp mediated F-actin dynamics.

Neural phenotypes of *sra-1* and *kette*

In addition to the CNS phenotypes, mutations in *sra-1* and *kette* both lead to synaptic defects that are characterized by an overall reduction in size of the neuromuscular junction, as well as the induction of supernumerary buds in *sra-1* and *kette* mutant synaptic boutons. It is known that new boutons often arise from existing ones by asymmetrical budding or symmetrical division (Zito et al., 1999), which in turn requires an intact regulation of the actin cytoskeleton (Luo, 2002; Notarangelo and Ochs, 2003). The increased number of branches as well as the bulged appearance of the synaptic boutons after depletion of *kette* or *sra-1* function may reflect their function in regulating *wasp* (Bogdan and Klämbt, 2003). Indeed *wasp* mutants display synaptic phenotypes similar to

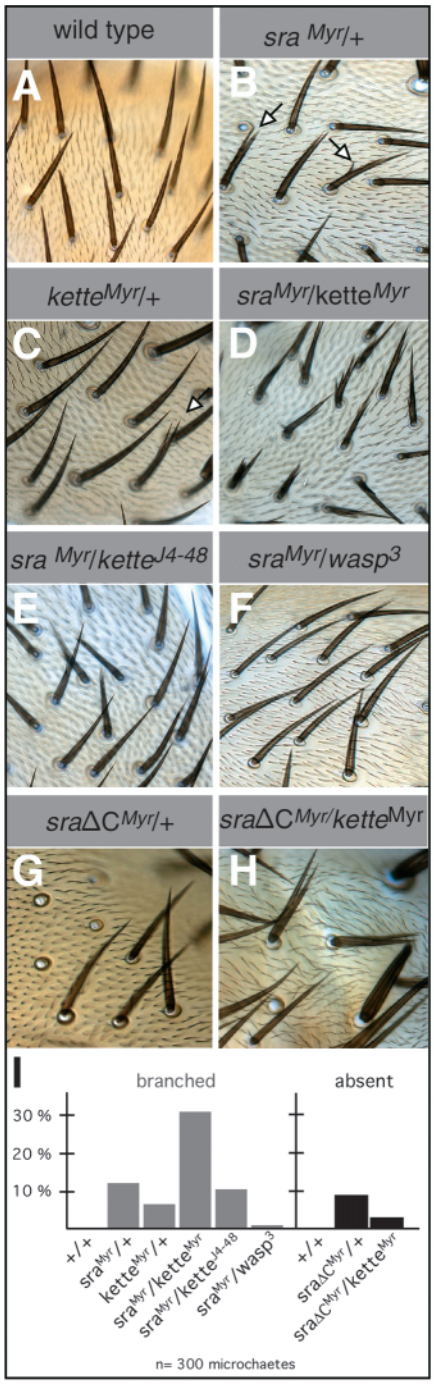

Fig. 8. Genetic interaction between *sra-1*, *kette* and *wasp*. (A-H) Morphology of microchaete on the thorax in different genetic backgrounds. Quantitative analyses are shown in I. (A) Wild-type flies are characterized by an ordered array of microchaete, which are normally thin, straight and have a pointed end. (B) Following expression of one copy of the UAS-Sra-1^{Myr} transgene in the *scabrous* pattern, shorter and split bristles develop (arrow). (C) The same phenotype is observed after expression of membrane tethered Kette protein (arrow). (D) Co-expression of both membrane-tethered Sra-1 and Kette leads to a synergistic increase of bristle defects. (E) Same genetic background as in B but lacking one copy of the *kette* gene. The bristle phenotype evoked by Sra-1^{Myr} expression is not affected. (F) Same genetic background as in B but lacking one copy of the *wasp* gene. The bristle phenotype evoked by Sra-1^{Myr} expression is suppressed. (G) Expression of a UAS-Sra-1ΔC^{Myr} transgene in the *scabrous* pattern results in loss of bristles. (H) When flies co-express UAS-Sra-1ΔC^{Myr} and UAS-Kette^{Myr} transgenes, the loss of bristle phenotype is suppressed. However, bristles are brushed, which corresponds to the Kette^{Myr} phenotype. (I) Quantitative analyses of the above-mentioned phenotypes. Three-hundred microchaete were counted in each case.

those of *sra-1* and *kette* (Coyle et al., 2004). Recently, it has been found that the adaptor protein Nervous wreck (Nwk) binds Wasp and is also required for normal synapse morphology (Coyle et al., 2004). Thus, Nwk might act as a scaffolding protein in the synapse assembling a Wasp activation complex comprising Sra-1, Kette and Abi.

As in *Drosophila*, mutations in several of the vertebrate orthologs of the above mentioned genes are associated with learning deficits, demonstrating the pivotal importance of F-actin dynamics for precise neuronal function (Eden et al., 2002; Endris et al., 2002; Soderling et al., 2003; Suzuki et al., 2000; Yamamoto et al., 2001).

Regulation of F-actin dynamics

Rapid remodeling of the F-actin cytoskeleton is mostly brought about by the Arp2/3 complex, which in turn is activated by members of the Wasp and Wave protein families (Machesky et al., 1994; Miki and Takenawa, 2003; Nakagawa et al., 2001; Takenawa and Miki, 2001). Wasp as well as Wave are potent F-actin nucleation factors. Obviously within the cell their activity must be tightly regulated. Whereas Wasp is auto-inhibited, Wave is trans-inhibited and requires the inhibiting Sra-1 Kette protein complex (Eden et al., 2002). Upon dissociation of this complex or conformational changes within the complex, Wave is active and presumably remains active until it is degraded via ubiquitination (Blagg et al., 2003; Eden et al., 2002; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004). This latter mechanism, which is frequently used in regulating the effective concentration of active proteins (Di Fiore et al., 2003; Moon et al., 2002; Ou et al., 2003), ensures that Wave activity lasts for only a short time period.

As we have shown here, Wave is not the only protein of the complex that is degraded upon disruption of the protein complex. Depletion of Kette not only leads to a loss of Wave but also of Sra-1. Vice versa, depletion of Sra-1 leads to a loss of Kette. Thus, ultimately the stability of all proteins of the inhibitory Sra-1 complex appears to be interdependent.

We have previously shown that Kette can activate Wasp-mediated F-actin formation (Bogdan and Klämbt, 2003). We have shown that Sra-1 function also depends on Wasp. In both cases, the membrane localization of Kette or Sra-1 is essential, indicating that in vivo regulation of membrane recruitment of Sra-1 and Kette is important for function. The data presented in this work also suggest that membrane-bound Sra-1 or Kette proteins are both able to activate Wasp independently of each other.

Membrane recruitment of Sra-1

Vertebrate homologues of Sra-1 and Kette were first identified in a complex with the SH2 SH3 adapter Nck (Kitamura et al., 1996; Kitamura et al., 1997). The N-terminal SH3 domain of Nck is thought to bind to Sra-1 (Kitamura et al., 1996), evoking a model where Nck recruits Sra-1 and the associated Kette

protein to the membrane. We have previously shown that *kette* and *dock*, which encodes the *Drosophila* Nck homologue interact during axonal pathfinding (Garrity et al., 1996; Hummel et al., 2000). Cell signaling and cell adhesion leads to the activation of a number of receptor systems which in turn mediate anchorage-dependent recruitment of adapter proteins such as Nck (Howe, 2001; Howe et al., 2002; Li et al., 2001). Nck in turn is able to connect cell-surface receptors via different signal cascades to the F-actin cytoskeleton (Buday et al., 2002).

However, in vivo Nck cannot mediate all aspects of Sra-1/Kette function, as the complete loss of maternal and zygotic Nck results in similar but not identical phenotypes when compared with *kette* or *sra-1* loss-of-function phenotypes (Fan et al., 2003; Garrity et al., 1996; Hummel et al., 2000; Schenck et al., 2003). In the developing synapse, the function of Nck in recruiting Sra-1 and Kette to the membrane may be fulfilled by Nwk, which as Nck also binds Wasp (Coyle et al., 2004; Zeng et al., 2003). Interestingly, both adaptor proteins are involved in Slit Robo signaling, where they may mediate different biological effects (Fan et al., 2003; Wong et al., 2001). This suggests that combinatorial and tissue specific factors are assembled in response to specific cues to activate Wasp in different cell types or compartments.

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