

Kidins220/ARMS regulates Rac1-dependent neurite outgrowth by direct interaction with the RhoGEF Trio

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Summary

Neurite extension depends on extracellular signals that lead to changes in gene expression and rearrangement of the actin cytoskeleton. A factor that might orchestrate these signalling pathways with cytoskeletal elements is the integral membrane protein Kidins220/ARMS, a downstream target of neurotrophins. Here, we identified Trio, a RhoGEF for Rac1, RhoG and RhoA, which is involved in neurite outgrowth and axon guidance, as a binding partner of Kidins220. This interaction is direct and occurs between the N-terminus of Trio and the ankyrin repeats of Kidins220. Trio and Kidins220 colocalise at the tips of neurites in NGF-differentiated PC12 cells, where F-actin and Rac1 also accumulate. Expression of the ankyrin repeats of Kidins220 in PC12 cells inhibits NGF-dependent and Trio-induced neurite outgrowth. Similar results are seen in primary hippocampal neurons. Our data indicate that Kidins220 might localise Trio to specific membrane sites and regulate its activity, leading to Rac1 activation and neurite outgrowth.

Key words: Neuronal differentiation, Neurotrophin signalling, Kidins220/ARMS, Rac1, Trio

Introduction

During neuronal development, axons and dendrites have to navigate long distances to reach their physiological targets. This process includes extensive membrane remodelling and neurite elongation, which depend on extracellular signals that prompt changes in gene expression and cytoskeletal rearrangements at the growth cone. Amongst other extracellular inputs, neurotrophins have major roles in mediating neuronal differentiation, survival and death (Huber et al., 2003; Lu et al., 2005). Nerve growth factor (NGF), one of the best-characterised neurotrophins, signals through the neurotrophin receptors TrkA and p75^{NTR}. Upon NGF binding, these receptors hetero-oligomerise and initiate pathways leading to sustained MAPK signalling, which is required for neurite outgrowth.

A molecule recently established to have a key role in sustained NGF-dependent MAPK signalling is Kidins220, also called ARMS (Arevalo et al., 2006; Arevalo et al., 2004; Hisata et al., 2007). Kidins220 is a highly conserved, integral membrane protein with large N- and C-terminal cytoplasmic domains (Iglesias et al., 2000; Kong et al., 2001). It acts as a downstream target of ephrin and neurotrophin receptors, such as p75^{NTR} and TrkA (Arevalo et al., 2004; Kong et al., 2001), with which it forms a ternary complex (Chang et al., 2004). Overexpression of dominant-negative Kidins220 mutants (Arevalo et al., 2006; Bracale et al., 2007), or its downregulation by siRNA (Arevalo et al., 2004; Hisata et al., 2007) causes a strong inhibition of sustained MAPK signalling and neurite outgrowth in PC12 cells.

In addition to changes in gene expression, rearrangements of the actin cytoskeleton are essential for neurite elongation. Members of the small RhoGTPase family regulate this process, with Rac1, Cdc42 and RhoG supporting neurite extension and RhoA mediating growth-cone retraction (Dickson, 2001; Govak et al., 2005). RhoGTPases cycle between an active, GTP-bound form and an inactive, GDP-bound form. The activation is under the control of Rho guanine nucleotide-exchange factors (RhoGEFs) specific for

a subset of RhoGTPases (Rossman et al., 2005), whereas they are switched off by their corresponding RhoGTPase-activating proteins (RhoGAPs).

Trio and its closely related family member Kalirin are recent additions to the growing family of RhoGEFs involved in neurite outgrowth. Both Trio and Kalirin possess two independent RhoGEF domains: the first (GEF1) is specific for Rac1 and RhoG, and the second (GEF2) is specific for RhoA (Bateman and Van Vactor, 2001; Bellanger et al., 1998; Blangy et al., 2000; Debant et al., 1996; Penzes et al., 2001). Trio has been implicated in axon guidance in a variety of organisms, such as *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*, via its first GEF domain (Awasaki et al., 2000; Bateman et al., 2000; Briancon-Marjollet et al., 2008; Liebl et al., 2000; Lin and Greenberg, 2000; Newsome et al., 2000; Steven et al., 1998). Trio-knockout mice die between embryonic day (E) 15.5 and birth (O'Brien et al., 2000). They display skeletal muscle deformity, aberrant cellular organisations in the brain (O'Brien et al., 2000) and axonal guidance defects, which relates to the involvement of Trio in netrin-1/DCC signalling (Briancon-Marjollet et al., 2008). Further studies show that Trio also regulates the organisation of neuronal clusters in the hindbrain in vivo (Backer et al., 2007).

In PC12 cells Trio is the rate-limiting factor of NGF-induced neurite outgrowth by the activation of RhoG via GEF1 (Estrach et al., 2002). This process is regulated by the N-terminal spectrin domains of Trio and its first SH3 domain (Estrach et al., 2002). Whereas the effect of the GEF1 of Trio on neurite outgrowth is easy to rationalise, the inhibition of NGF-induced neurite outgrowth observed by expressing the spectrin repeats and the first SH3 domain is still lacking a precise explanation.

To date, the mechanisms implicated in neurotrophin signalling down to the re-organisation of the actin cytoskeleton are only partially understood. Therefore, factors capable of receiving and integrating extracellular signals, and at the same time, modulating the actin

cytoskeleton, could have a key role in orchestrating signalling events and actin rearrangements that lead to neurite outgrowth. Kidins220 is ideally placed to fulfil this function by acting as a scaffolding protein for neurotrophin receptors (Arevalo et al., 2004; Kong et al., 2001) and providing docking sites for modulators of the actin cytoskeleton. Here, we show that the N-terminal ankyrin repeats of Kidins220 bind directly to the spectrin domains of the RhoGEF Trio. Overexpression of the ankyrin-rich domain of Kidins220 leads to enhanced Rac1 activation and to impairment of Trio- and NGF-induced neurite elongation. Altogether, these data suggest that the interaction between Trio and Kidins220 has an important role in neurite outgrowth in PC12 cells and primary neurons.

Results

Interaction between Kidins220 and Trio

To explore the role of Kidins220 in neurite elongation, we searched for possible interacting partners by a yeast two-hybrid approach using the N-terminal domain of Kidins220 as bait. This intracellular domain consists of 11 ankyrin repeats (1-402 aa) (Fig. 1A), but does not include the Walker A motif (467-474 aa), which, together with the Walker B (771-775 aa), forms a P-loop NTPase domain. The N-terminal 672 residues of the RhoGEF Trio, which contains the Sec14 and its first four spectrin domains (Fig. 1B), were among the positive hits (for a complete list of putative interactors, see supplementary material Table S1). This interaction was tested by re-transforming yeast, either with the N-terminal domain of Kidins220 (1-402 aa; Kidins220¹⁻⁴⁰²) or with its C-terminus (1209-1762 aa; Kidins220¹²⁰⁹⁻¹⁷⁶²) together with Trio¹⁻⁶⁷². Only yeast co-transformed with the ankyrin repeats of Kidins220 and the N-terminus of Trio showed robust growth (supplementary material Fig. S1A), hence confirming the result of the yeast two-hybrid screen.

Next, we verified the interaction of Kidins220 with Trio by co-immunoprecipitation experiments of endogenous proteins in PC12 cells. Anti-Trio (Fig. 1C) and anti-Kidins220 antibodies (Fig. 1D) were able to specifically co-immunoprecipitate Kidins220 and Trio, respectively. This interaction took place both in the absence and in the presence of NGF (Fig. 1E), suggesting that the interaction of Trio and Kidins220 is largely constitutive, as was previously observed for the ternary complex formation between Kidins220 and the neurotrophin receptors p75^{NTR} and TrkA (Chang et al., 2004). The efficiency of the Trio-Kidins220 co-immunoprecipitation was comparable with other known interactors of Kidins220, such as p75^{NTR} (Chang et al., 2004) or kinesin-1 (Bracale et al., 2007).

Because of the high sequence similarity between Trio and its closely related family member Kalirin, we decided to test whether Kidins220 also interacted with Kalirin. Although we did not detect an interaction between endogenous Kidins220 and Kalirin, Kalirin9 weakly co-immunoprecipitated with Kidins220 in PC12 cells transfected with HA-tagged Kidins220 (Fig. 1F). Interestingly this Kalirin isoform was previously shown to interact with p75^{NTR} and activate RhoA (Harrington et al., 2008). However, since we found Trio in the yeast two-hybrid screen and we were able to co-immunoprecipitate both endogenous Trio and Kidins220, we focussed our investigation on this specific interaction.

To map the determinants of the interaction of Trio with Kidins220, co-immunoprecipitation experiments were performed with the indicated GFP-tagged Trio constructs (Fig. 1B,G). PC12 cells were transfected, treated with NGF for 24 hours and extracts were immunoprecipitated with anti-Kidins220 antibodies. As shown in Fig. 1G, the fragment containing the spectrin repeats of Trio

(Trio¹⁻¹²⁰³) was immunoprecipitated, whereas the other domains of Trio, including the large C-terminus (Trio¹⁸¹³⁻³⁰³⁸), were not. This association was specific, because it was not detected when this lysate was incubated with a rabbit pre-immune serum (pre-I) used as negative control. Because the band of GFP-tagged Trio¹⁵⁷⁹⁻¹⁸¹⁶ ran at a similar molecular mass to the immunoglobulin heavy chain, anti-Kidins220 antibodies and pre-immune serum coupled to protein-G-Sepharose were loaded on the same gel (Fig. 1G and supplementary material Fig. S1B). Comparison of these lanes showed that the contaminating bands, indicated by black and grey arrowheads, had slightly different mobility than GFP-tagged Trio¹⁵⁷⁹⁻¹⁸¹⁶.

To test whether the interaction between Kidins220 and Trio was direct, the His₆-tagged N-terminal ankyrin-rich domain of Kidins220 was expressed and purified. This fragment was subsequently incubated with either GST alone or the GST-tagged Sec14 domain (Trio¹⁻²³²), spectrin domains 1-4 (Trio²⁵⁵⁻⁶⁹⁹) and 5-8 (Trio⁶⁹⁶⁻¹²⁰³) (supplementary material Fig. S1C). Both spectrin clusters, and to a lesser extent the Sec14 domain, bound to Kidins220¹⁻⁴⁰² (Fig. 1H), indicating that this interaction is direct and that the N-terminus of Trio, including all spectrin domains, participates in the formation of the Trio-Kidins220 complex.

Kidins220 and Trio colocalise at neurite tips

The binding of Kidins220 to Trio suggests that Kidins220 affects actin cytoskeleton dynamics. If this were the case, Kidins220 should localise, at least in part, to regions enriched in actin microfilaments. To test this hypothesis, PC12 cells were differentiated for 3 days with NGF, then fixed and stained for F-actin using fluorescently labelled phalloidin, and for endogenous Kidins220. As shown in Fig. 2A,B, F-actin and Kidins220 showed an extensive overlap at neurite tips (arrows). This enrichment of Kidins220 at neurite tips was not due to an accumulation of plasma membrane or cytoplasm at these sites, because Kidins220 and a membrane-bound version of mRFP (mRFP-CAAX; Fig. 2C) or soluble GFP (Fig. 2D) did not show the same cellular distribution (arrowheads). Interestingly, Trio also localises to actin-rich regions at growth cones (Estrach et al., 2002), a finding that prompted us to directly compare the localisation of Trio and Kidins220. Because anti-Trio antibodies do not recognise the endogenous protein in immunofluorescence, GFP-tagged full length Trio was expressed in NGF-differentiated PC12 cells, which were then immunostained for Kidins220. Both proteins could be detected at the tip of neurites (Fig. 2E,F), indicating that the interaction between Trio and Kidins220 takes place on extending neurites and growth cones.

What is the molecular function of the Kidins220-Trio complex? Since Kidins220 is an integral membrane protein and interacts directly with Trio, it might drive the recruitment of the latter, a mainly cytosolic protein, to specific membrane domains. To test this hypothesis, we overexpressed HA-tagged Kidins220 in PC12 cells and tested whether the cellular localisation of Trio is influenced by full-length Kidins220. Owing to the absence of suitable anti-Trio antibodies for immunofluorescence (see above) and the very low transfection efficiency of full-length Trio, we used a truncated Trio construct containing all spectrin repeats, the first GEF and SH3 domain (Fig. 1B). This Trio fragment (Trio¹⁻¹⁸¹³) was shown to mimic full-length Trio in promotion of NGF-induced neurite outgrowth in PC12 cells (Estrach et al., 2002). Since GFP-tagged Trio¹⁻¹⁸¹³ is partially cytoplasmic as judged by immunofluorescence (Fig. 3A), co-expression of this fragment with full-length Kidins220

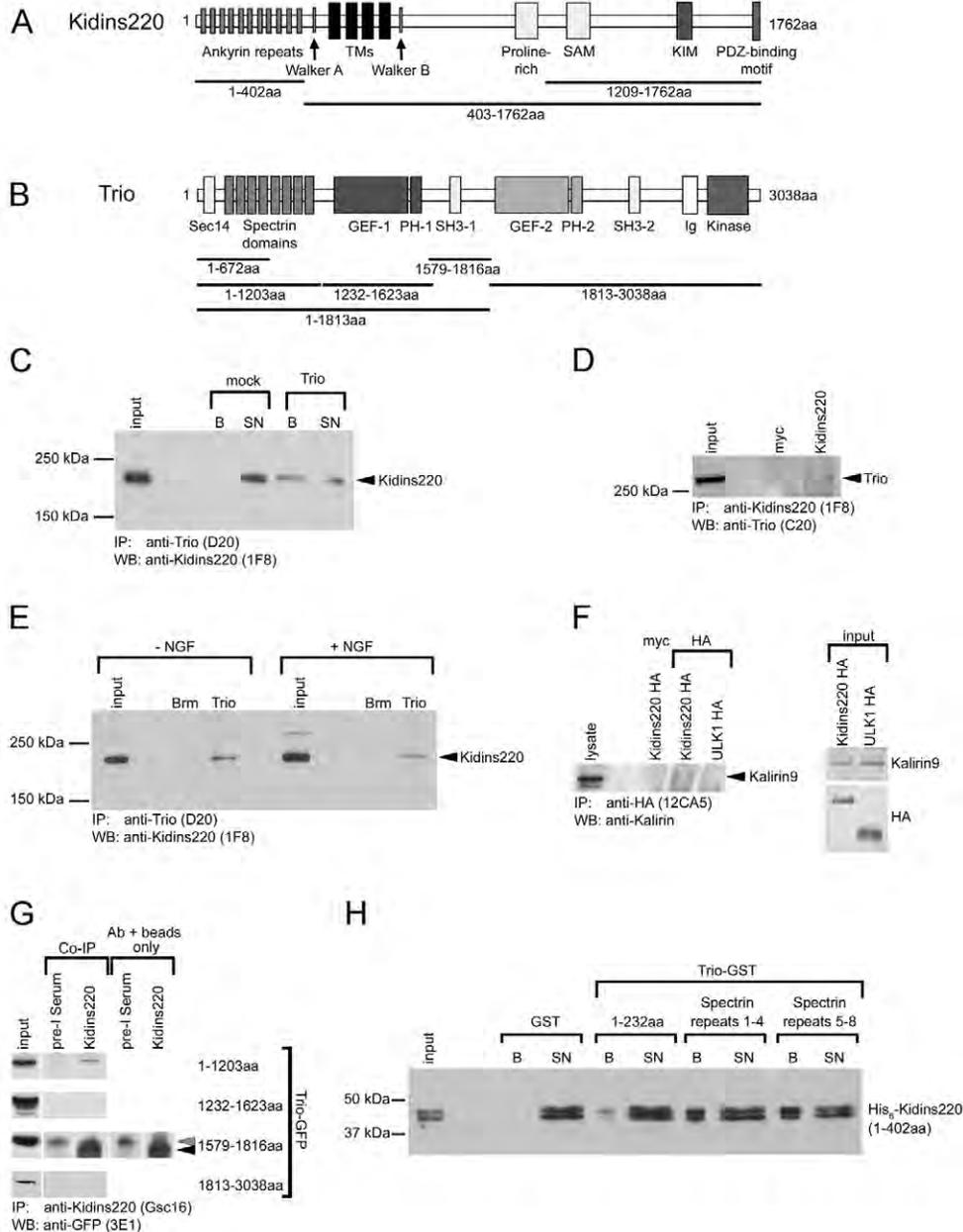


Fig. 1. Interaction between Kidins220 and Trio. (A) Kidins220 is an integral membrane protein containing four transmembrane domains (TMs) and large N- and C-termini exposed to the cytoplasm. At the N-terminus, primary sequence analysis predicts 11 ankyrin repeats (1-402 aa) and a Walker A motif (467-474 aa), which together with the Walker B motif (771-775 aa) forms a P-loop NTPase domain. The C-terminus contains a proline-rich domain, a sterile alpha motif (SAM)-like domain and a PDZ-binding motif. Kidins220 binds kinesin light chain via the kinesin-1-interacting motif (KIM). Several phosphorylation sites are also present (not shown). The Kidins220 fragments used in this study are underlined. (B) Trio is a 348 kDa protein, comprised of a putative Sec14-like domain, followed by eight spectrin repeats (255-1203 aa), two Rho-GEF and SH3 domains, an immunoglobulin (Ig)-like domain and a kinase domain at the C-terminus. The Trio fragments used in this study are underlined. (C,D) The interaction between Kidins220 and Trio was confirmed by co-immunoprecipitation of both endogenous proteins. NGF-treated PC12 cell extracts were immunoprecipitated with anti-Trio (C) or anti-Kidins220 antibodies (D). Western blots were probed with anti-Kidins220 and anti-Trio antibodies, respectively. In both experiments, endogenous Kidins220 and Trio were recovered in the pellet. (E) The association of Trio and Kidins220 is not modulated by NGF. To test whether the Kidins220-Trio interaction was NGF-dependent, PC12 E2 cells – a PC12 cell clone with enhanced sensitivity to neurite outgrowth promoting factors (Estrach et al., 2002; Wu and Bradshaw, 1995) – were incubated either with or without NGF for 24 hours, lysed and then immunoprecipitated with anti-Trio antibodies. (F) PC12 cells were transfected with HA-Kidins220 and HA-ULK1 as negative control, and then immunoprecipitated with anti-HA antibodies. The western blot was probed with anti-Kalirin antibodies, which revealed a weak band of Kalirin9 in the Kidins220 lane, but not in control immunoprecipitations. (G) To map the binding domains of Trio, PC12 cells were transfected with GFP-Trio constructs and lysates were immunoprecipitated with anti-Kidins220 antibodies. Immunoglobulin-contaminating bands of the anti-Kidins220 antibodies and of the pre-immune serum (Pre-I) are indicated with black and grey arrowheads, respectively. Only the spectrin domains of Trio (Trio¹⁻¹²⁰³) are immunoprecipitated by Kidins220 antibodies. (H) The ankyrin repeats of Kidins220 and the N-terminal fragments of Trio (Trio¹⁻²³², Trio²⁵⁵⁻⁶⁹⁹, Trio⁶⁹⁶⁻¹²⁰³) were expressed as recombinant proteins and tested for direct binding. The ankyrin repeats (Kidins220¹⁻⁴⁰²) bind to a small extent to the Sec14 domain (Trio¹⁻²³²), but interact equally well with the first (Trio²⁵⁵⁻⁶⁹⁹, repeats 1-4) and the second half of the spectrin domains (Trio⁶⁹⁶⁻¹²⁰³, repeats 5-8). SN, supernatant; B, beads.

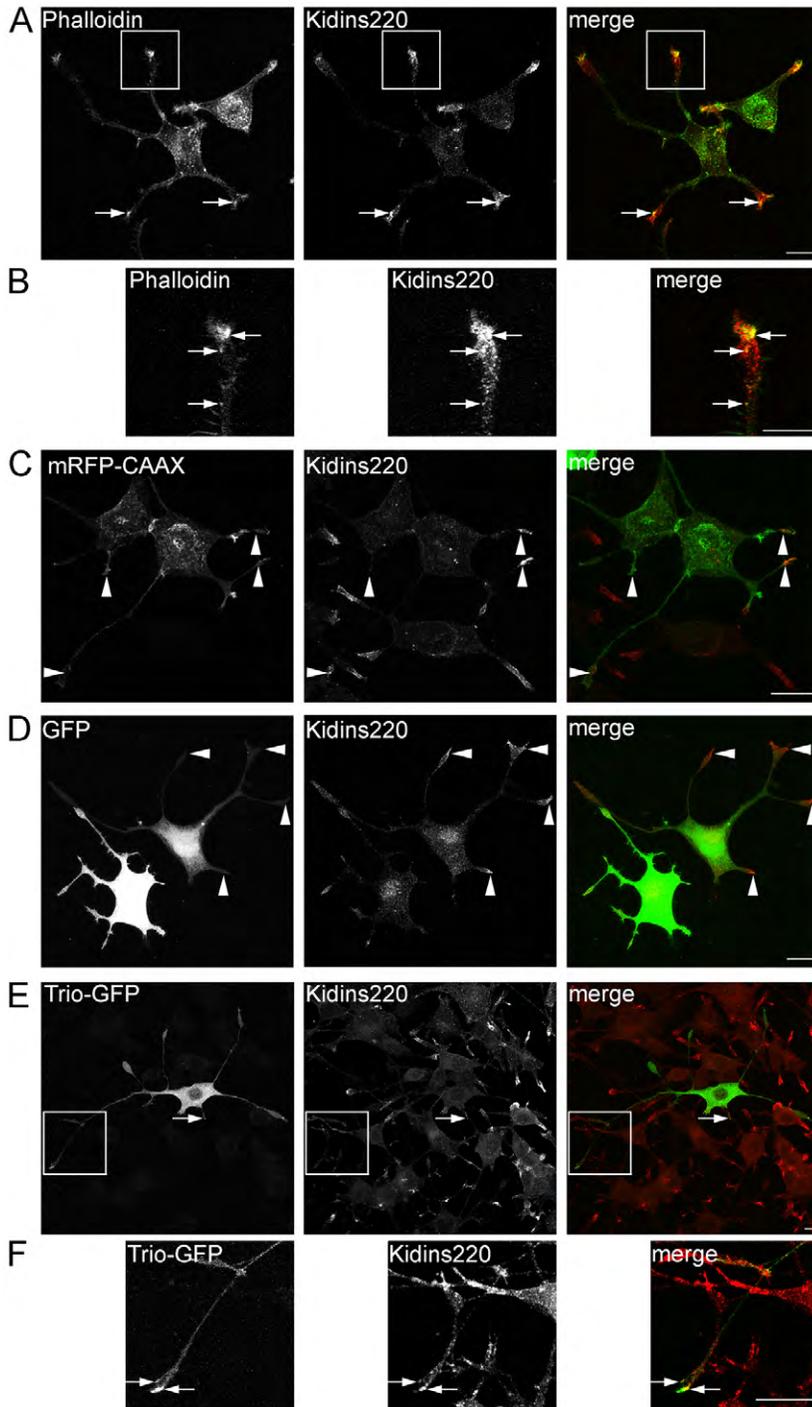


Fig. 2. Colocalisation of Kidins220 and Trio. (A) NGF-differentiated PC12 cells were stained with phalloidin (green) and Kidins220 (red). Both markers overlap partially at the neurite tips (arrows). (B) High magnification view of a neurite tip. PC12 cells were transfected with a membrane-bound mRFP construct (mRFP-CAAX) (C) or soluble GFP (D), differentiated with NGF and stained for endogenous Kidins220. Arrowheads indicate the different cellular distribution of these proteins. (E) PC12 cells were transfected with Trio-GFP (green), differentiated with NGF and then immunostained for endogenous Kidins220 (red). Both proteins colocalised partially at neurite tips (arrows). (F) High magnification view. Scale bars: 5 μ m.

determined its recruitment onto Kidins220-positive membrane-bound puncta (double-positive structures=50.3 \pm 7.4%) (Fig. 3B,C; arrows). This effect was not observed in cells co-expressing full-length Kidins220 and GFP (double-positive structures=5.3 \pm 1.2%) (Fig. 3D, arrowheads). In contrast to full-length Kidins220, expression of the membrane-anchored Kidins220⁴⁰³⁻¹⁷⁶², which lacks the Trio-binding domain, was not able to localise Trio¹⁻¹⁸¹³ to the membrane (double-positive structures=8.7 \pm 3.3%) (Fig. 3E,F; arrowheads), indicating that full-length Kidins220 might help to localise Trio to specific membrane domains and as a consequence, regulate its cellular activities.

Kidins220¹⁻⁴⁰² activates Rac1 but not RhoG

To investigate the functional effects of a Trio-Kidins220 complex, we tested whether Kidins220 would activate downstream effectors of Trio. Since NGF-induced neurite outgrowth is mediated by its first GEF domain (Estrach et al., 2002), we looked at the activation of Rac1 and RhoG, both of which can be activated by this domain. Full-length Kidins220, the Trio-interacting ankyrin repeats (Kidins220¹⁻⁴⁰²) and the remaining C-terminal portion of Kidins220 (Kidins220⁴⁰³⁻¹⁷⁶²) were expressed as HA-tagged proteins in HEK293 cells (see Materials and Methods and supplementary material Fig. S2A). Cell lysates were then examined for the amount

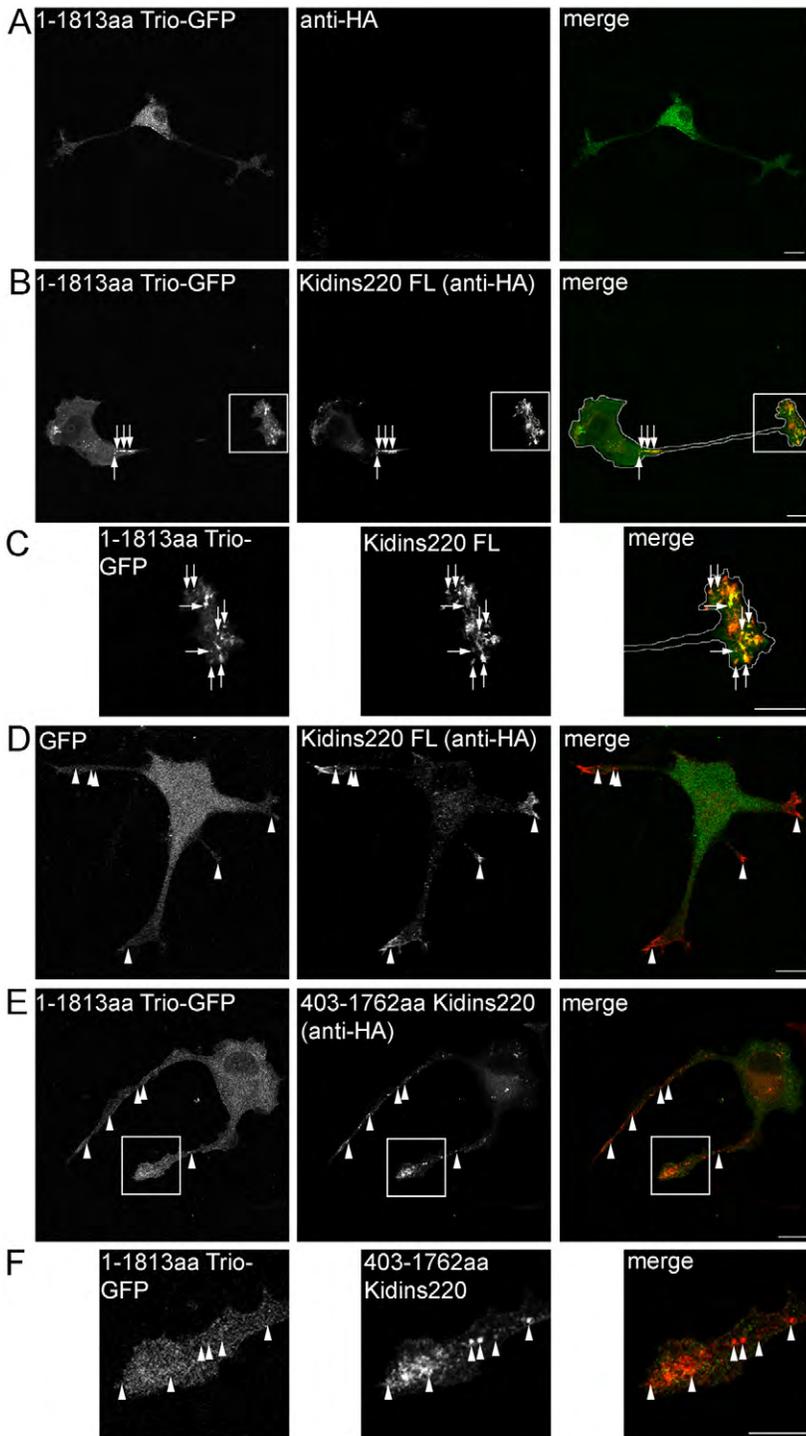


Fig. 3. Trio associates with Kidins220-positive membrane structures. (A,B) PC12 cells were transfected with GFP-tagged Trio¹⁻¹⁸¹³ and HA-tagged full length Kidins220, differentiated for 3 days with NGF, fixed and analysed by immunofluorescence. GFP-tagged Trio¹⁻¹⁸¹³ is partially diffused (A), but it is recruited to Kidins220-positive puncta upon co-expression with full-length Kidins220 (B, arrows). (C) A high-magnification view of the neurite tip in B. Arrows indicate colocalising puncta. The transfected cell and the growth cone are outlined for better visibility. (D) Co-expression of GFP and HA-tagged full-length Kidins220 did not result in a recruitment of GFP to Kidins220-positive puncta (arrowheads). (E) PC12 cells were co-transfected with GFP-tagged Trio¹⁻¹⁸¹³ and HA-tagged Kidins220⁴⁰³⁻¹⁷⁶² and treated as in B. GFP-tagged Trio¹⁻¹⁸¹³ does not associate with Kidins220⁴⁰³⁻¹⁷⁶²-positive structures (arrowheads). (F) An enlargement of the neurite tip in E. FL, full length. Scale bars: 5 μm.

of activated, GTP-bound Rac1 using a GST-pull-down assay performed with the Cdc42/Rac1 interacting-binding (CRIB) domain of the Rac1 effector protein Pak1. Overexpression of Kidins220¹⁻⁴⁰², and to a small but significant extent of full-length Kidins220, led to a reproducible activation of Rac1 (Fig. 4A,B), suggesting a possible regulation of the TrioGEF1 activity by Kidins220.

However, these experiments did not address whether the Rac1 activation by the Kidins220-Trio complex was direct or was due to the activation of RhoG, which in turn could activate Rac1 via

the DOCK180-ELMO complex (Kato and Negishi, 2003). To test this, we performed a RhoG activation assay with the GST-tagged kinectin domain 1 (KID1) of the RhoG effector kinectin (Vignal et al., 2001), following a protocol similar to that described above for the Rac1 activation. For the detection of endogenous RhoG, we used a monoclonal anti-RhoG antibody, which does not crossreact with Rac1 (Meller et al., 2008). HEK293 cells were transfected with HA-tagged Kidins220 constructs, with GFP as negative and TrioGEF1 as positive controls (supplementary material Fig. S2C). As expected, TrioGEF1 potentially activated endogenous

RhoG (Fig. 4C). However, transfection of full-length Kidins220 or Kidins220¹⁻⁴⁰² did not affect the levels of GTP-bound RhoG (Fig. 4C,D), indicating that Rac1 activation by the Kidins220-Trio complex is direct and not mediated by RhoG.

Since Kidins220 was able to activate Rac1 via Trio, we assessed the subcellular localisation of both proteins in differentiated PC12 cells. A pool of Rac1 localised to neurite tips and the staining of Kidins220 and Rac1 overlapped in these areas (Fig. 4E, arrows).

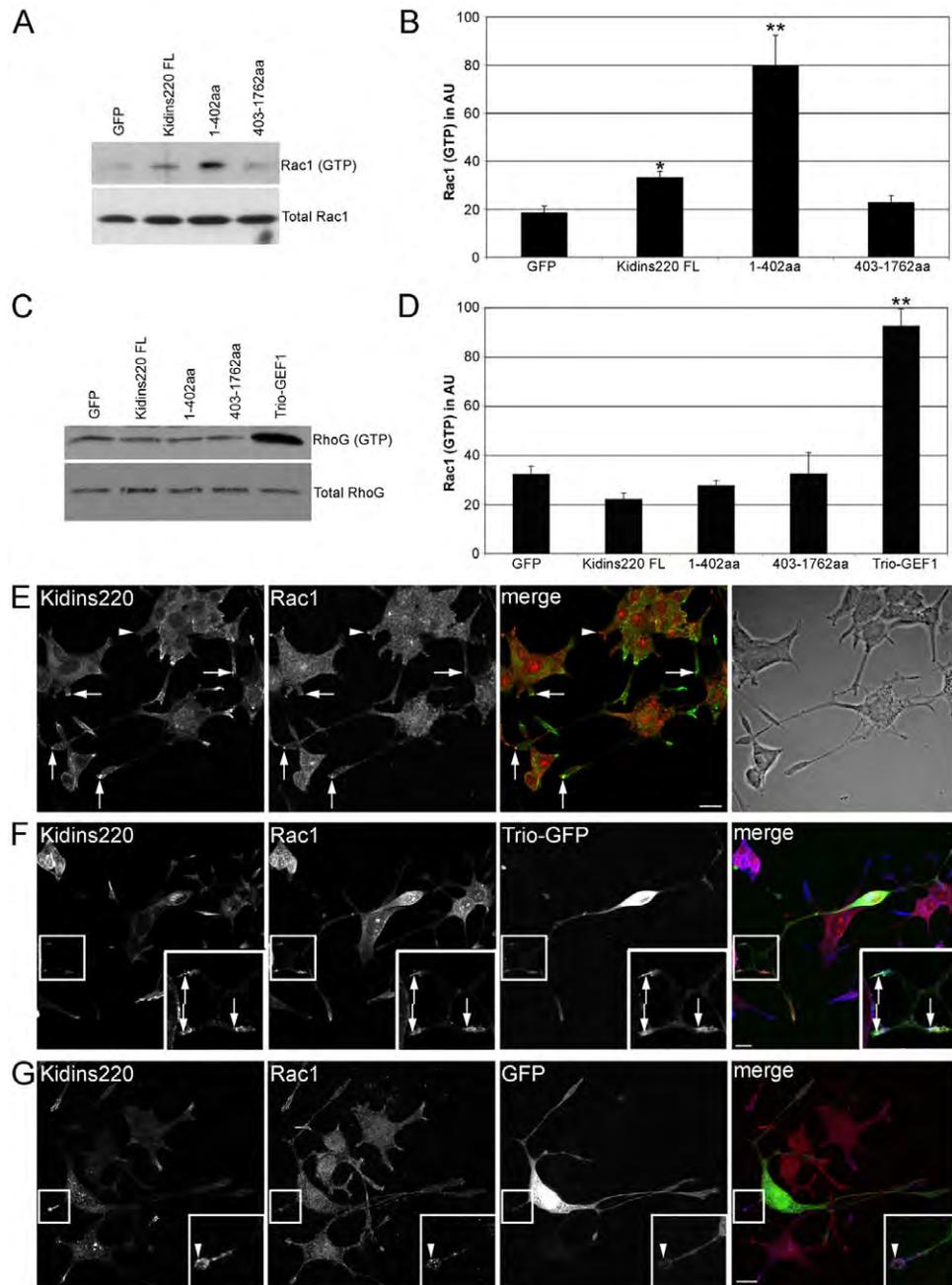


Fig. 4. Kidins220 expression activates Rac1 but not RhoG. (A) HEK293 cells were transfected with the indicated constructs and subjected to a Rac1 activation assay. Cells transfected with Kidins220¹⁻⁴⁰² and to a lesser extent with full-length Kidins220 show an increase in Rac1-GTP levels. (B) Quantification of three independent experiments. Rac1-GTP levels were expressed in arbitrary units (AU), after they had been normalised to the expression of each construct and to the total Rac1 content. The activation of Rac1 by Kidins220¹⁻⁴⁰² and full-length protein is significant in the Student's *t*-test; * $P \leq 0.05$; ** $P \leq 0.01$. Error bars represent s.e.m. (C) HEK293 cells were transfected with the indicated constructs and subjected to a RhoG-activation assay. The first GEF domain of Trio activated endogenous RhoG, but none of the other constructs did. (D) The means of three independent experiments. Error bars represent s.e.m. FL, full length; ** $P \leq 0.01$. (E) PC12 cells were differentiated for 3 days with NGF, then fixed and stained for endogenous Kidins220 (green) and Rac1 (red). Arrows indicate overlapping puncta and arrowheads show nonoverlapping areas. (F) Trio-GFP was expressed in NGF-differentiated PC12 cells, which were subsequently fixed and immunostained for endogenous Kidins220 (blue) and Rac1 (red). (G) Positive structures for all three proteins are observed at neurite tips (white, arrows), whereas soluble GFP is distributed rather uniformly throughout the cell. The arrowhead indicates a Kidins220/Rac1-positive structure, which was not enriched in GFP. Scale bars: 5 μ m.

However, Kidins220 and Rac1 did not colocalise on all neurite protrusions (Fig. 4E, arrowheads), reflecting the possibility that not all neurites undergo dynamic changes at any given time, and some of them might even undergo retraction. Consistently, Rac1 is transiently activated at the cell periphery and neurite tips of NGF-differentiated PC12 cells, and this event is required for neurite outgrowth (Aoki et al., 2004). Next, we transfected GFP-tagged Trio in PC12 cells, and after 3 days of differentiation with NGF, the localisation of endogenous Rac1 and Kidins220 was revealed by immunostaining. As shown in Fig. 4F, the three proteins colocalised at some neurite tips: sites where they might actively promote neurite extension. This localisation is specific for Trio-GFP, because GFP was not enriched at these sites (Fig. 4G, arrowhead).

The ankyrin repeats of Kidins220 inhibit NGF-induced neurite outgrowth

Given the importance of Rac1 and Trio in neurite outgrowth, we wondered whether overexpression of Kidins220 and its fragments would affect this process. We decided to pursue this strategy, instead of Kidins220 downregulation by RNA interference, since the latter approach, which has been previously shown to inhibit neurite outgrowth in PC12 cells (Hisata et al., 2007), would only provide insights on the overall function of Kidins220, and would not allow us to distinguish between the role of its interaction with Trio or with other binding partners (Arevalo et al., 2006; Arevalo et al., 2004; Bracale et al., 2007; Hisata et al., 2007; Iglesias et al., 2000; Kong et al., 2001; Luo et al., 2005). Therefore, we analysed the extent of NGF-induced differentiation in PC12 cells after overexpression of Kidins220¹⁻⁴⁰², Kidins220⁴⁰³⁻¹⁷⁶² and the full-length protein, whilst GFP was used as a negative control (Fig. S3A). Cells were treated for 3 days with NGF, fixed, and the neurite length of transfected cells was measured. Cells bearing at least one neurite twice the length of their cell body were scored as positive. Overexpression of the N-terminal fragment, but not of Kidins220⁴⁰³⁻¹⁷⁶² or full-length Kidins220, inhibited neurite outgrowth (Fig. 5A, supplementary material Fig. S3A). As shown in supplementary material Fig. S3B,C, the ankyrin-rich Kidins220¹⁻⁴⁰² domain is expressed less strongly than Kidins220⁴⁰³⁻¹⁷⁶² or Kidins220¹⁻¹⁷⁶², indicating that its inhibitory effect on neurite outgrowth (20.7±2.2% differentiated cells versus 69±6.9% for GFP-transfected cells; Fig. 5A) is not due to massive overexpression.

Based on these results, we wondered how overexpression of Kidins220¹⁻⁴⁰², which was able to drive Rac1 activation (Fig. 4A), could at the same time inhibit neurite outgrowth. As seen by immunofluorescence, the ankyrin repeats were distributed evenly throughout the cell (supplementary material Fig. S3A, inset). Therefore, an attractive possibility is that Rac1 activation by Kidins220¹⁻⁴⁰² occurs ubiquitously in transfected cells, instead of at specific sites on the plasma membrane, which is a prerequisite for neurite formation (Aoki et al., 2004; da Silva and Dotti, 2002). In this light, Kidins220¹⁻⁴⁰² overexpression would mimic the inhibitory effect on NGF-dependent neurite outgrowth induced by overexpression of a constitutively active Rac1 mutant (Aoki et al., 2004). To test this hypothesis, we used the Raichu construct Rac1/Rac1-C-terminus (CT) as a biosensor to monitor Rac1 activation in live cells. This probe monitors the balance of activity between GEFs and GAPs acting on Rac1 and was previously used to study the localisation of activated Rac1 in NGF-treated PC12 cells (Aoki et al., 2004). We co-transfected this biosensor with

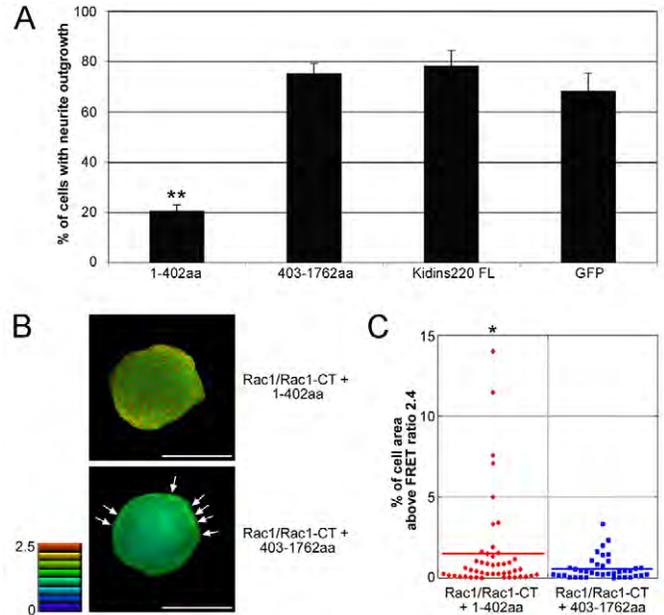


Fig. 5. Kidins220¹⁻⁴⁰² overexpression inhibits NGF-dependent neurite outgrowth. (A) PC12 cells were transfected with GFP or the indicated HA-tagged fragments of Kidins220, differentiated with NGF for 3 days and then analysed for the length of their neurites. Neurite outgrowth was quantified by counting cells bearing neurites at least twice as long as their cell body. Quantification was obtained from three independent experiments (\pm s.e.m.). At least 62 cells were analysed per condition. FL, full length. $**P \leq 0.01$. (B) PC12 cells were co-transfected with the Raichu Rac1/Rac1-CT and HA-tagged Kidins220¹⁻⁴⁰² (upper panel) or Kidins220⁴⁰³⁻¹⁷⁶² (lower panel). The FRET ratio is shown in intensity-modulated display (IMD) format, ranging from a FRET ratio 0 to 2.5. High FRET ratios are observed inside the cell after co-transfection of Kidins220¹⁻⁴⁰². Scale bars: 10 μ m. (C) For quantification, the cell area (in % of the whole cell) occupied by a FRET ratio of 2.4 and above was summed (see the Materials and Methods for details). These values were then compared between cells co-expressing the Raichu construct with Kidins220¹⁻⁴⁰² (red circles) or Kidins220⁴⁰³⁻¹⁷⁶² (blue squares). Each symbol represents an individual cell. The red and blue bars indicate the mean of 49 Kidins220¹⁻⁴⁰²-Raichu Rac1/Rac1-CT co-transfected cells or 42 Kidins220⁴⁰³⁻¹⁷⁶²-Raichu Rac1/Rac1-CT co-transfected cells, respectively. Cells expressing Kidins220¹⁻⁴⁰² exhibit more cell area occupied by high FRET ratios. $*P \leq 0.05$.

HA-tagged Kidins220¹⁻⁴⁰² or Kidins220⁴⁰³⁻¹⁷⁶² in PC12 cells and examined the localisation of activated Rac1. To this end, the FRET ratio between the emission intensities of acceptor and donor was measured in living cells. In cells co-transfected with Kidins220¹⁻⁴⁰² we observed activation of Rac1 throughout the cell (Fig. 5B, upper panel). By contrast, cells co-transfected with the C-terminus of Kidins220, which we used as a negative control, showed lower levels of GTP-bound Rac1 that partially localised along the plasma membrane (Fig. 5B, lower panel, arrows). Aoki and colleagues devised an elegant way of measuring cell areas with a FRET ratio above a certain threshold, which allows testing of whether a localised activation of Rac1 is required for neurite outgrowth (Aoki et al., 2004). They could use as a reference the same cell before NGF treatment, whereas in our experiments, which involve two cell populations transfected with different constructs, we had to empirically set a threshold ratio above which we could detect a difference in Rac1 activation (see the Materials and Methods). We observed a significant difference in

the cell area occupied by activated Rac1 in cells cotransfected with Kidins220¹⁻⁴⁰² or Kidins220⁴⁰³⁻¹⁷⁶² at a FRET ratio above 2.4 (Fig. 5C). This result correlates well with our biochemical data (Fig. 4A,B). Cells transfected only with Raichu Rac1/Rac1-CT displayed an intermediate value (data not shown). At present we are unable to assess whether this difference is due to an inhibitory effect of the C-terminus of Kidins220, or whether the transfection of the Raichu construct alone results in a higher basal activation of Rac1.

Taken together, these results suggest that the ankyrin repeats of Kidins220 triggered a ubiquitous activation of Rac1. Since a localised activation of Rac1 at specific sites at or close to the plasma membrane is necessary for neurite extension, overexpression of Kidins220¹⁻⁴⁰² determined a net inhibition in NGF-induced neurite outgrowth (Fig. 5A).

Effect of Kidins220 on Trio- and RhoG-induced neurite elongation

To study more specifically the functional consequences of the interaction between Kidins220 and Trio, we looked at the effect that Kidins220 and its deletion mutants might have on Trio-induced neurite outgrowth. To this end, PC12 E2 cells, a PC12 cell clone with enhanced sensitivity to factors promoting neurite outgrowth (Estrach et al., 2002; Wu and Bradshaw, 1995), were double-transfected with GFP-Trio¹⁻¹⁸¹³ and either mRFP, full-length Kidins220 or its deletion mutants. As in Fig. 3, we used the C-terminal truncated version of Trio (Trio¹⁻¹⁸¹³). After transfection,

cells were incubated for 48 hours in full growth medium, then fixed and analysed for their neurite length as described above. Under these conditions, expression of full-length Kidins220 alone induced a minor increase (9.2±1.6%) in neurite outgrowth, compared with 4.9±1.2% in mRFP expressing cells ($P=0.077$) and to 3.6±0.9% in Kidins220¹⁻⁴⁰² expressing cells ($P=0.023$) (Fig. 6A). In co-transfection experiments, only double-expressing cells were examined for neurite outgrowth, as described earlier (supplementary material Fig. S4). Cells double transfected with Trio¹⁻¹⁸¹³ and mRFP showed neurite outgrowth in 35.3±4.7% of cases, representing the baseline for Trio-induced neurite outgrowth (Fig. 6C; $n=3$ independent experiments). Expression of full-length Kidins220 or Kidins220⁴⁰³⁻¹⁷⁶² did not alter Trio-induced neurite elongation, whereas expression of Kidins220¹⁻⁴⁰² significantly inhibited this process (Fig. 6C). Since Kidins220¹⁻⁴⁰² is partially cytosolic (Fig. 6F), we wondered whether a membrane-bound variant of Kidins220¹⁻⁴⁰² would exert the same effect. Therefore we added a CAAX box at the C-terminus of Kidins¹⁻⁴⁰²: a modification that has previously been shown to determine prenylation and recruitment of soluble proteins to the membrane. As shown in Fig. 6F, CAAX-tagged Kidins220¹⁻⁴⁰² was bound to the membrane pellet. However, it was still able to reduce Trio-induced neurite outgrowth (Fig. 6D), indicating that this effect is independent of the membrane association of Kidins¹⁻⁴⁰². Its inhibitory characteristic might rather be due to the fact that this mutant does not localise to neurite tips (supplementary material

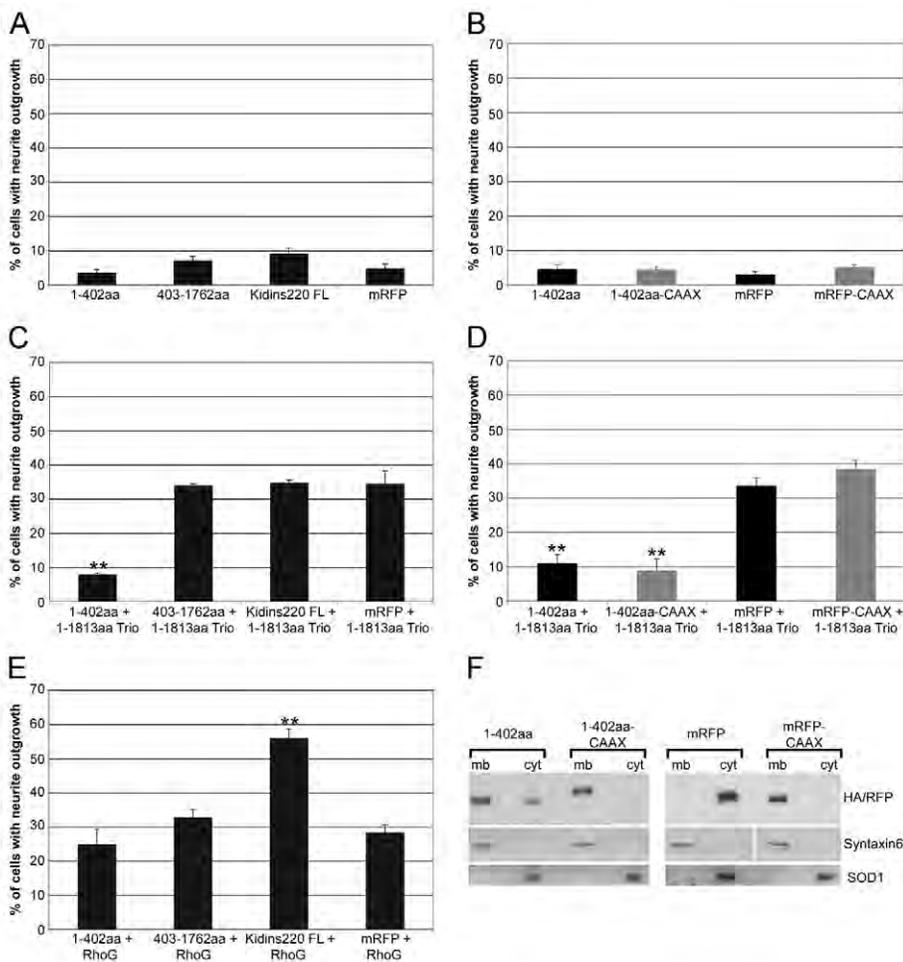


Fig. 6. Quantification of Trio and RhoG-induced neurite outgrowth. (A,B) PC12 E2 cells were transfected with mRFP, mRFP-CAAX or HA-tagged Kidins220 deletion mutants. After 48 hours expression in full growth medium, cells were fixed and examined for neurite outgrowth. Cells, which bore one neurite at least double the size of the diameter of its cell body, were considered to be positive ($n \geq 3$ independent experiments). PC12 E2 cells were co-transfected either with GFP-tagged Trio¹⁻¹⁸¹³ (C,D) or RhoG (E) and the indicated HA-tagged Kidins220 deletion mutants or mRFP and mRFP-CAAX as negative controls. After 48 hours, double-transfected cells were quantified for neurite outgrowth in three independent experiments, as previously described. Kidins220¹⁻⁴⁰² and the membrane-bound Kidins220¹⁻⁴⁰²-CAAX blocked Trio-induced neurite elongation (C,D), whereas full-length Kidins220 enhanced RhoG-induced neurite outgrowth (E). Error bars represent s.e.m. At least 154 cells were analysed for each construct. FL, full length; ** $P \leq 0.01$. (F) HEK293 cells were transfected with HA-tagged Kidins220¹⁻⁴⁰², Kidins220¹⁻⁴⁰²-CAAX, mRFP or mRFP-CAAX and subjected to a membrane-cytosol fractionation. Syntaxin6 was used as a membrane marker and SOD1 as a marker for the cytosol. Whereas the ankyrin repeats are partially cytosolic, Kidins220¹⁻⁴⁰²-CAAX and mRFP-CAAX associate with membrane fractions.

Fig. S5, inset), where Rac1 should be activated to promote neurite outgrowth.

RhoG, another downstream target of Trio, is also able to promote neurite elongation when overexpressed (Estrach et al., 2002; Katoh et al., 2000). Since Kidins220¹⁻⁴⁰² led to the specific activation of Rac1 and not RhoG, we anticipated that overexpression of the ankyrin repeats should not affect the RhoG-induced neurite outgrowth. Therefore, PC12 E2 cells were double transfected with wild-type RhoG-GFP and either mRFP or Kidins220 deletion mutants for 48 hours (supplementary material Fig. S6). Neither the N- nor C-terminal domains of Kidins220 altered neurite outgrowth induced by RhoG (Fig. 6E; $n=3$ independent experiments). These results confirm our biochemical evidence that the ankyrin repeats of Kidins220 did not promote RhoG activation. However, co-expression of full-length Kidins220 with wild-type RhoG doubled the number of cells bearing neurites (Fig. 6E), indicating that full-length Kidins220 and RhoG might synergise with each other.

The N-terminus of Kidins220 inhibits neurite outgrowth in primary neurons

To confirm the role of the Trio-Kidins220 interaction in neurite elongation, we tested the effect of Kidins220¹⁻⁴⁰² in primary neurons. For this experiment, we chose hippocampal neurons, because Trio-knockout mice displayed abnormal neural organisation in the hippocampus (O'Brien et al., 2000) and Kidins220 is highly expressed in this region of the brain (Kong et al., 2001; Wu et al., 2009). Therefore E18 hippocampal neurons were nucleofected with Kidins220 deletion mutants or GFP (as a negative control). After 1 day *in vitro*, neurons were fixed and neurite length was measured (Fig. 7A-C). Under these conditions, GFP-expressing hippocampal neurons had a normal appearance, with the majority of cells showing one or more neurites (Fig. 7A,D). Neurons expressing Kidins220⁴⁰³⁻¹⁷⁶² also showed neurites at this stage (Fig. 7C,D). However, neurons overexpressing Kidins220¹⁻⁴⁰² were not able to develop neurites (Fig. 7B,D). Because of their rounded appearance, we were concerned that these cells might be necrotic or apoptotic. Therefore we performed a co-staining with propidium iodide, which labels the nucleus of necrotic cells when added before fixation. In addition, nucleofected neurons were treated with an antibody specific for the activated form of caspase 3, an early apoptosis marker. These tests revealed that neurons expressing Kidins220¹⁻⁴⁰² were not necrotic (Fig. 7Bb, green) or apoptotic (Fig. 7Bb, red). Unfortunately, we were not able to express full-length Kidins220 in neurons, because of its propensity to aggregate upon overexpression. Since most hippocampal neurons expressing Kidins220¹⁻⁴⁰² did not bear neurites after 1 day *in vitro*, it is likely that the ankyrin repeats affects neurite initiation, which is important for both dendritic and axonal growth. This is in accordance with previous studies, which showed that Kidins220 regulates dendritic branching *in vivo* (Wu et al., 2009), and modulated the development of both axons and dendrites (Higuero et al., 2009). In summary, this experiment demonstrates that the inhibitory activity of the ankyrin-rich domain of Kidins220 on neurite outgrowth also occurs in hippocampal neurons, strongly suggesting that the pathway elucidated in PC12 cells has general validity and controls differentiation in primary neurons.

Discussion

We analysed the interaction of the scaffolding molecule Kidins220 with the RhoGEF Trio and ascertained its functional consequences. Mapping of the interaction sites between these two proteins

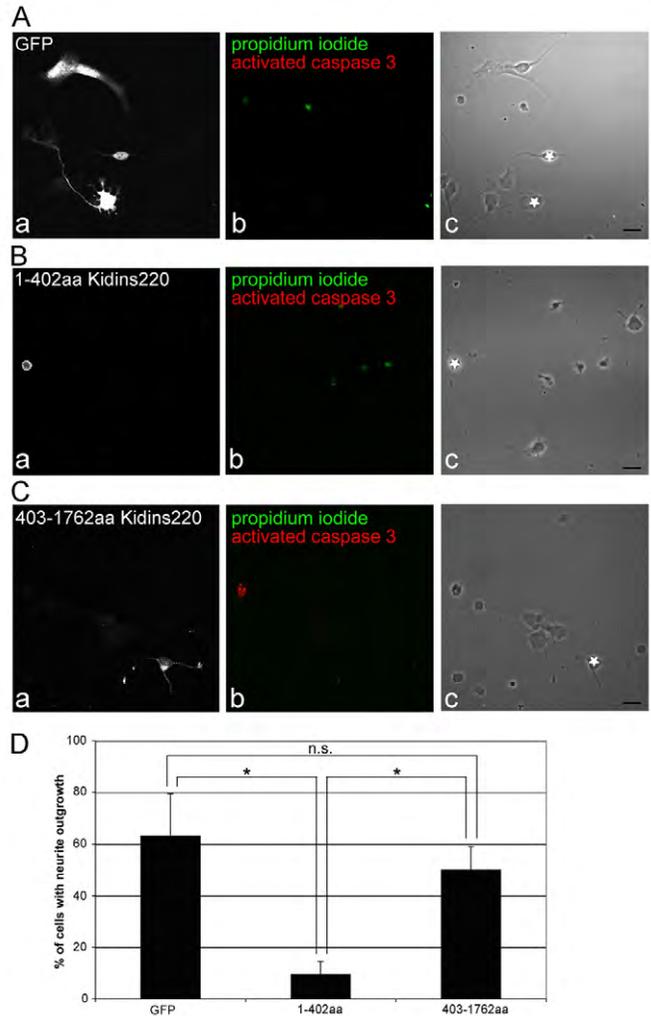


Fig. 7. Kidins220¹⁻⁴⁰² overexpression inhibits neurite outgrowth in hippocampal neurons. (A-C) Primary neurons were nucleofected with GFP or the indicated HA-tagged deletion mutants of Kidins220. After 1 day, they were fixed and analysed for the length of their neurites. Transfected neurons are indicated with stars. To detect necrotic or apoptotic cells, neurons were co-stained with propidium iodide (green) or antibodies against activated caspase 3 (red) (panels b). Scale bars: 10 μ m. (D) The average of four independent experiments. Neurons transfected with Kidins220¹⁻⁴⁰² display impaired neurite outgrowth compared with control cells. Error bars represent s.e.m. At least 227 neurons were analysed for each construct. * $P \leq 0.05$; n.s., not significant.

revealed that the N-terminal ankyrin-rich domain of Kidins220 (Kidins220¹⁻⁴⁰²) bound directly to the N-terminal spectrin repeats of Trio. Both proteins colocalised at the tips of neurites in NGF-differentiated PC12 cells, where F-actin and Rac1 also accumulated. Rac1, but not RhoG, was specifically activated by Kidins220¹⁻⁴⁰² and, to a small extent, by the full-length protein. Strikingly, overexpression of the ankyrin repeats of Kidins220 inhibited neurite outgrowth, whereas its C-terminus had no effect.

Because expression of Kidins220¹⁻⁴⁰² specifically inhibited both NGF- and Trio-induced neurite outgrowth, and NGF-mediated outgrowth depends on Trio (Estrach et al., 2002), it is likely that the dominant-negative effect elicited by Kidins220¹⁻⁴⁰² is due to its interaction with the spectrin-rich domain of Trio. Our results might therefore explain the observed inhibitory effect of the latter domain

on NGF-dependent differentiation (Estrach et al., 2002). The inhibition of neurite outgrowth by Kidins220¹⁻⁴⁰² is not a general effect, because its overexpression did not affect neurite extension induced by RhoG (Fig. 6E). This is in agreement with our biochemical data that Kidins220 did not activate RhoG. However, full-length Kidins220 increased RhoG-induced neurite outgrowth (Fig. 6E). Because both RhoG and Kidins220 can activate Rac1 independently (Fig. 8A; pathway A and B), contributions from these two signalling cascades could occur in parallel, and thus their effects on neurite outgrowth might be additive. By contrast, full-length Kidins220 was not able to synergise with Trio in promoting neurite elongation (Fig. 6C), suggesting that they act on the same pathway (Fig. 8A; pathway B), with Trio being rate-limiting.

We observed a selective activation of Rac1, but not of RhoG, by the ankyrin repeats of Kidins220. Given the direct interaction between Kidins220 and Trio that we report here, we propose that the activation of Rac1 is mediated by Trio. This selectivity for Rac1 is not unprecedented. In fact, DCC/Netrin-induced neurite outgrowth mainly uses the Trio-dependent Rac1 pathway and a dominant-negative form of RhoG did not interfere with this process (Briancon-Marjollet et al., 2008). In addition, Backer and colleagues reported that Trio controls the organisation of neuronal clusters in the hindbrain via Rac1 and not RhoG (Backer et al., 2007), although previous reports show that Rac1 can be activated by RhoG via the DOCK180-ELMO complex (Katoh and Negishi, 2003). Since the TrioGEF1 domain is able to activate both RhoG and Rac1 (Bellanger et al., 1998; Blangy et al., 2000; Chhatriwala et al., 2007), one attractive possibility is that it is the specific process that determines whether RhoG or Rac1 is activated. It was previously reported that RhoG, but not Rac1, mediates the membrane association of Trio, since Trio alone was not able to do so (Skowronek et al., 2004). In our system, Trio might be recruited to the membrane with the help of Kidins220 (Fig. 8A), because Kidins220 is an integral membrane protein and its binding to Trio must occur at specific membrane sites. In this scenario, Rac1 could be activated by Trio without an activation of RhoG and its downstream targets, such as Cdc42 (Gauthier-Rouviere et al., 1998).

The finding that the expression of Kidins220¹⁻⁴⁰² activated Rac1 and inhibited neurite outgrowth phenocopies the effects of expressing constitutively-active Rac1, which also leads to a reduction of neurite outgrowth in PC12 cells (Aoki et al., 2004). A possible explanation for this phenomenon is that Rac1 needs to be activated at specific sites, such as neurite tips, to sustain neurite extension (Aoki et al., 2004). However, Kidins220¹⁻⁴⁰² is not localised at these sites, allowing a ubiquitous activation of Rac1 (Fig. 5B) and consequently a block of differentiation (Fig. 8B, left). Full-length Kidins220, which also activated Rac1 although to a very small extent, did not prevent neurite elongation, most likely because it is correctly localised to neurite tips similarly to endogenous Kidins220 (Fig. 8B, right).

The phenotypic differences observed by expressing full-length Kidins220 and its ankyrin-rich domain do not only originate from their different cellular localisation, but might also derive from the fact that Kidins220 consists of two functionally distinct N- and C-terminal domains (see Fig. 1A), whose crosstalk is probably essential to exert its function as a scaffolding protein (Fig. 8B; right). In contrast to the full-length protein, Kidins220¹⁻⁴⁰² is unable to receive any information from the C-terminal portion of Kidins220, which is known to have a key role in the binding of neurotrophin receptors and contains several protein-protein interaction domains (Fig. 1A) (Arevalo et al., 2006; Arevalo et al.,

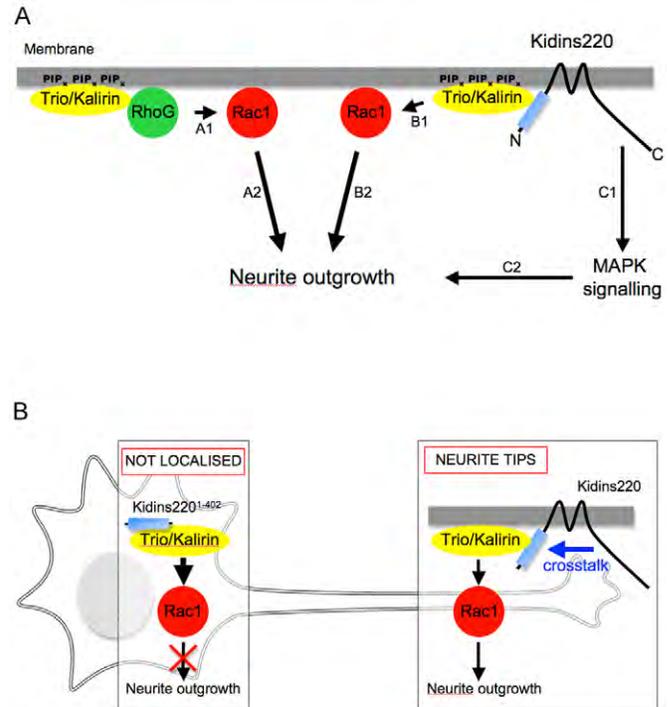


Fig. 8. Schematic view of the Kidins220-Trio interaction in a cellular context. (A) Kidins220 is a transmembrane protein that can assist in the recruitment of Trio (and/or Kalirin) to the membrane. Here, Rac1 might be activated, leading to neurite outgrowth (pathway B). At the same time, the C-terminus of Kidins220 is phosphorylated upon NGF stimulation and is involved in sustained MAPK signalling, which is also necessary for neurite outgrowth (pathway C). In parallel, Trio might translocate to the membrane with the help of RhoG (pathway A). This leads to activation of Rac1 and might explain the enhancement of neurite outgrowth when Kidins220 and RhoG are overexpressed. Trio and RhoG can bind to a variety of phospholipids (PIP_x) (Skowronek et al., 2004; Ugolev et al., 2008). (B) The different phenotypes generated by the expression of the ankyrin-rich Kidins220¹⁻⁴⁰² domain and the full-length protein is likely to be due to their different localisations. Kidins220¹⁻⁴⁰² is distributed throughout the cell and activates Rac1, leading to an inhibition of neurite outgrowth similar to constitutively active Rac1 (Aoki et al., 2004). By contrast, full-length Kidins220 is localised to neurite tips and is likely to determine the activation of Rac1 at these specific sites. Moreover, in full-length Kidins220, the C-terminus integrates information received from upstream signals, such as ephrins and neurotrophins, which could be transmitted to the N-terminus.

2004; Bracale et al., 2007; Hisata et al., 2007; Iglesias et al., 2000; Kong et al., 2001; Luo et al., 2005). Crucially, the C-terminal domain undergoes tyrosine phosphorylation upon neurotrophin stimulation, and this event is required for the recruitment of downstream effectors necessary for sustained MAPK signalling (Arevalo et al., 2006) (pathway C in Fig. 8A).

Kidins220 might also exert its function through other Trio-related proteins. This view is supported by preliminary data showing that the inhibition of neurite outgrowth mediated by Kidins220¹⁻⁴⁰² overexpression is still observed in neurons derived from Trio-knockout mice (data not shown) (O'Brien et al., 2000), and by the interaction between Kidins220 and Kalirin, a Trio family member. Altogether, these results suggest that the functions of Trio and Kalirin might overlap. This is not surprising, since in *C. elegans* only one member of the Trio/Kalirin family, called Unc-73, is expressed (Bateman and van Vactor, 2001), suggesting that multiple

roles for Trio and Kalirin were acquired with the increased complexity of the organisms. Interestingly, Kidins220 interacts with the TrkB receptor and is phosphorylated upon treatment with its ligand, brain-derived neurotrophic factor (BDNF) (Arevalo et al., 2004; Kong et al., 2001; Luo et al., 2005). In addition, Kidins220 is a downstream target of ephrins (Kong et al., 2001; Luo et al., 2005), which similarly to TrkB, control the morphology of dendritic spines (Ethell et al., 2001; Gorski et al., 2003; Horch and Katz, 2002; Horch et al., 1999; Klein, 2004; Penzes et al., 2003). Accordingly, Kidins220 is enriched at the postsynaptic density (Wu and Chao, personal communication), and dendritic-spine stability is significantly reduced in *Kidins220^{-/-}* mice (Wu et al., 2009). It is interesting in this context that Kalirin has a major role in dendritic-spine morphogenesis (Cahill et al., 2009; Ma et al., 2003; Ma et al., 2008; Penzes et al., 2003; Penzes and Jones, 2008), suggesting a new connection by Kidins220 between these pathways.

In this light, the interaction of Kidins220 with Trio or other Trio/Kalirin family members is likely to be crucial to control neuronal differentiation and functions *in vivo*. The work presented here therefore might describe a basic molecular mechanism underlying this process. Thus, the scaffolding protein Kidins220 possesses all the prerequisites to co-ordinate different input signals from neurotrophins and ephrins with cytoskeletal rearrangements controlled by Trio/Kalirin and Rac1.

Materials and Methods

Plasmids

GFP-tagged Trio and RhoG were previously described (Estrach et al., 2002), as were the GST-tagged CRIB domain of Pak1 and the KID1 domain of kinectin (Briançon-Marjolle et al., 2008) and Vignal et al. (Vignal et al., 2001). The Raichu construct Rac1/Rac-CT containing the coding sequence of Rac1, Rac1 C-terminus and the CRIB domain of Pak1 flanked with GFP reporters was a gift from Michiyuki Matsuda (Aoki et al., 2004; Yoshizaki et al., 2003). This construct was cloned into the pCAGGS expression vector, provided by Jun Miyazaki (Niwa et al., 1991).

DNA constructs

Kidins220 1-1206bp (1-402 aa) and 1207-5289bp (403-1762 aa) were amplified by PCR using rat full-length *Kidins220* cDNA (Iglesias et al., 2000) and the following primers: Kidins220¹⁻⁴⁰² 5' primer, cat tca ceg cgg gg tca gtt ctt ata tca cag ag and 3' primer, cg cgt ggg ctt aat taa tca ttt gtt ggg tct gta gag; Kidins220⁴⁰³⁻¹⁷⁶² 5' primer, c att ata ceg cgg gg gca ggc gag act ccc tac and 3' primer, gc ggg cgt taa tta aag aat gct ctc tct ctc). DNA fragments were then ligated into the *PacI*-*SacII* sites of a HA-tagged pcDNA3.1 vector (Invitrogen). GST-tagged fragments of Trio (1-232 aa, 255-699 aa and 696-1203 aa) were subcloned from GFP-tagged Trio constructs (Estrach et al., 2002) into the *EcoRI* and *Sall* sites of pGEX4T2 (GE Biotech). Constructs used in the yeast two-hybrid analysis were subcloned into the *NdeI*-*NotI* sites of pGBKT7 (bait), or the *EcoRI*-*XhoI* sites of pGADT7 (prey), both from Clontech. For the preparation of Kidins220¹⁻⁴⁰²-CAAX, the stop codon in HA-Kidins220¹⁻⁴⁰² was first deleted by site-directed mutagenesis (TGA to TCG), using the 5' primer, tac aga ccc aac aaa tgc tta att aat tgc aat c and the 3' primer, gat tgc aat taa tta acc att tgt tgg gtc tgc a. The CAAX box of the mRFP-CAAX plasmid [a kind gift from Steven Hooper and Erik Sahai (Gaggioli et al., 2007)], was amplified by PCR with the 5' primer, cgc gcc tta att aac aag atg agc aag gac and the 3' primer, cgc gcc gca tgc att tac ata att aca cac ttt g, digested with *PacI* and *Clal* and ligated at the 3' end of the modified HA-Kidins220¹⁻⁴⁰² plasmid. All clones were verified by DNA sequencing.

Cell culture and transfection

PC12 cells or PC12 E2 cells (Wu and Bradshaw, 1995) were cultured in DMEM (Gibco) supplemented with 7.5% fetal bovine serum (FBS) and 7.5% horse serum (HS), or 5% FBS and 10% HS, respectively. HEK293 cells were cultured in DMEM with 10% FBS. Cells were transfected using Lipofectamine 2000 (Invitrogen) and when indicated, treated with 100 ng/ml NGF (Alomone) for 3 days in DMEM with 0.1% FBS and 0.1% HS. Since the expression of Kidins220¹⁻⁴⁰² was low, the quantity of DNA for each construct was optimised to achieve similar expression levels in the RhoGTPase activation assays (supplementary material Fig. S2A).

Hippocampal neurons were prepared from E18 embryos. 200,000 cells were transfected using the Amaxa nucleofector Small Cell Number Kit according to manufacturer's instructions and plated on coverslips coated with poly-L-lysine and laminin (Sigma) in DMEM, 10% HS. Subsequently, the medium was changed to

Neurobasal Medium (Gibco) with 2% B27 (Invitrogen). 10 minutes before fixation, neurons were treated with propidium iodide, fixed and immunostained as described below.

Cells were analysed for neurite outgrowth by dividing the length of their longest neurite by the diameter of their cell body using ImageJ (National Institutes of Health). If this ratio was higher than two, cells were scored as positive. For the quantification of the experiment in Fig. 3, ten cells per condition were analysed for Kidins220¹⁻¹⁷⁶² and Kidins220⁴⁰³⁻¹⁷⁶² co-transfected with GFP-tagged Trio¹⁻¹⁸¹³ and six cells were analysed for Kidins220¹⁻¹⁷⁶² and GFP double-expressing cells. In total, 320 Kidins220¹⁻¹⁷⁶² and 416 Kidins220⁴⁰³⁻¹⁷⁶² puncta were counted and scored positive when they also contained Trio¹⁻¹⁸¹³. 310 Kidins220¹⁻¹⁷⁶²-positive puncta were similarly analysed in GFP double-transfected cells.

Antibodies and immunostaining

Fixation, immunofluorescence staining and image acquisition were performed as previously described (Bracale et al., 2007). In Fig. 2, cells are shown as projections of confocal stacks, whereas in Figs 3 and 4 individual confocal stacks are presented. The following primary antibodies were used: mouse anti-Kidins220 (Bracale et al., 2007), rabbit anti-Kidins220 (Iglesias et al., 2000), goat anti-Trio C20 and D20 (Santa Cruz), mouse anti-Rac1 (clone 102/Rac1, BD Biosciences) and mouse anti-Rac1 (clone 23A8, Millipore), mouse anti-RhoG (clone 1F3B3E5, Santa Cruz), rat anti-HA (clone 3F10, Roche), mouse anti-HA (clone 12CA5, CRUK), rabbit anti-RFP (Abcam), rabbit anti-syntaxin6 (STO112, CRUK), rabbit anti-SOD1 (Abcam), rabbit anti-Kalirin (US Biological), mouse anti-GFP (clone 3E1, CRUK), rabbit anti-activated caspase 3 (RD Systems), mouse anti-myc (clone 9E10, CRUK), goat anti-Brm (Santa Cruz) and mouse anti-RGS-His₆ (Qiagen) antibodies. Alexa-Fluor-488-, -546- and -647-conjugated secondary antibodies were from Molecular Probes.

FRET analysis of Rac1 activation

The Raichu construct Rac1/Rac1 CT (Aoki et al., 2004) was co-transfected with HA-tagged Kidins220¹⁻⁴⁰² or Kidins220⁴⁰³⁻¹⁷⁶² into PC12 cells plated on grid glass-bottom dishes (Ibidi). After 1 day, images were taken on an inverted Nikon Eclipse TE 2000-E microscope equipped with a Xenon lamp, an iXon^{EM} CCD camera (Andor Technology) using a 100× oil-immersion DIC objective with 1.4 N.A. Filters (T455LP, CFP dichroic; 51017bs, CFP/YFP dichroic; 430/24X and 500/20X, excitation filters; 470/24M and 533/30M, emission filters) were from Chroma. Images were acquired with Metamorph Software (Universal Imaging) in imaging medium (DMEM without Phenol Red, folic acid and riboflavin, but supplemented with 7.5% FBS and 7.5% HS) at 37°C and 10% CO₂. Each cell was imaged in the CFP, FRET (CFP excitation/YFP emission) and YFP channel to calculate the FRET ratio (FRET/CFP). After imaging, cells were fixed and immunostained with an anti-HA antibody followed by an Alexa-Fluor-647-conjugated secondary antibody to identify double-transfected cells on a Zeiss confocal microscope. Bleedthrough between channels was assessed by expressing γ -BAR-CFP and γ -BAR-YFP (Neubrand et al., 2005), and acquiring images using the same settings as optimised for the Raichu construct (1×1 binning, 500 msecond exposure time for the CFP and FRET channel, 250 mseconds for the YFP channel). The measured bleedthrough of the CFP into FRET channel was 47±0.75% corresponding to the CFP emission spectra. Since the correction for the CFP bleedthrough in the FRET channel results in the FRET ratio minus a constant [(FRET/CFP)-0.47], and in this experiment these two conditions were directly compared with each other, this correction was neglected. The bleedthrough of the YFP channel into the FRET channel was assessed as a constant value of 20±0.45% for different expression levels, and the formula above was corrected to (FRET-0.2YFP)/CFP. After background subtraction, images were analysed using this formula in Metamorph (Universal Imaging). Resulting images were used to evaluate the FRET efficiency. In addition, expression levels of the Raichu construct were assessed in the YFP channel in each cell. For quantification purposes, only cells with expression levels between 500 and 5000 grey levels in a 16-bit image were considered. A ratio between 0 and 4 with a binning of 10 grey levels in an 8-bit image was applied to each cell. Since we were unable to detect a significant difference between treated and control cells by simply averaging the FRET ratio over the whole cell, we chose the following strategy similar to the one proposed by Aoki and co-workers (Aoki et al., 2004). The cell area, which was occupied by a certain FRET ratio, was expressed as a percentage of the whole cell. Subsequently, the sum of these percentages above a certain threshold ratio was used to compare cell populations co-transfected with the Raichu construct and Kidins220¹⁻⁴⁰² or Kidins220⁴⁰³⁻¹⁷⁶². At the FRET ratio of 2.4, the highest significant value between cells co-transfected with Kidins220¹⁻⁴⁰² and Kidins220⁴⁰³⁻¹⁷⁶² was reached, as assessed with the Student's *t*-test.

His₆-tagged Kidins220 protein purification

A DNA fragment encoding 1-402 aa of Kidins220 was inserted into a M15pQE31 vector (Qiagen) to generate a His₆-tagged fusion protein. Protein expression was induced with 0.4 mM IPTG. Bacteria were grown for additional 3.5 hours at 25°C, pelleted, resuspended in 25 mM Tris-HCl, pH 7.8, 300 mM KCl, 2 mM 2-mercaptoethanol and protease inhibitors and lysed using a French press. After centrifugation, the pellet was resuspended in 8 M urea in PBS containing 0.05% Tween and after removal of cell debris, the supernatant was incubated with Ni-NTA agarose (Qiagen) at 25°C for 45 minutes. Beads were washed initially with 100 mM

Tris-HCl, pH 8.0, 6 M urea, 20 mM imidazole, following by a gradient in which the concentration of urea was slowly brought to 0. The His₆-tagged protein was eluted with 500 mM imidazole and dialysed against 20 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 0.1 M urea and 5% glycerol.

Western blotting and immunoprecipitation

For immunoprecipitation, PC12 cells were lysed with IP buffer [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 1 mM EDTA and protease inhibitors (Roche)]. Lysates (1 mg per sample) were precleared with protein-G-Sepharose (GE Biotech), centrifuged at 15,000 g and the supernatant was incubated for 3 hours with 10 µg anti-Trio D20 or anti-Kidins220 (Gsc16 or 1F8) antibodies. A mouse anti-Myc antibody, a goat anti-Brm antibody, a rabbit-pre-immune serum or no antibody (mock) was included as negative control, depending on the species of the immunoprecipitating antibody. Immunocomplexes were recovered with protein-G-Sepharose overnight, washed with PBS and 0.1% Nonidet-P40 (supplemented with 500 mM NaCl for Fig. 1E,G), and then prepared for SDS-PAGE. Horseradish peroxidase-conjugated secondary antibodies (DakoCytomation) and ECL (GE Biotech) were used for detection.

GST-protein purification and direct-binding assay

GST fusion proteins corresponding to different Trio fragments were expressed in *E. coli* BL21 (DE3) cells. 1 litre of log phase cells was induced with 1 mM IPTG and shaken at 22°C overnight. Bacteria were lysed in 20 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 µg/ml lysozyme, 1 µg/ml DNase), sonicated, and centrifuged at 138,000 g at 4°C for 20 minutes. The clarified lysate was mixed at 4°C for 1 hour with glutathione-Sepharose 4B (GE Biotech) equilibrated with 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100. Beads were washed four times with this buffer, once with 50 mM Tris-HCl, pH 8.0, and then used in the binding assay.

The binding of His₆-tagged Kidins220¹⁻⁴⁰² to various GST fusion proteins was assayed in binding buffer (25 mM HEPES-NaOH, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM 2-mercaptoethanol, 0.1% Triton X-100), as described previously (Neubrand et al., 2005), using 30 µg of the purified GST fusion proteins and 16.6 µg/ml of His₆-tagged Kidins220¹⁻⁴⁰² in 0.9 ml. Briefly, samples were incubated for 1 hour at 4°C and beads were recovered by centrifugation. After 4 washes with binding buffer, 1/10th of each pellet and 1/60th of each supernatant were subjected to SDS-PAGE and western blotting. Blots were probed with anti-His₆ antibodies (Qiagen), and detected by ECL.

RhoGTPase activation assays

For the Rac1 activation assay, a GST-pulldown using the CRIB domain of Pak1 was performed (Briancon-Marjollet et al., 2008). *E. coli* B21 bacteria transformed with a pGEX-vector encoding GST-CRIB were grown at 37°C until they reached an OD₆₀₀ of 0.6. Protein expression was induced by IPTG (0.1 mM final concentration) and cultures were further incubated for 3 hours at 20°C. Bacteria were harvested by centrifugation at 6000 g for 15 minutes at 4°C, resuspended in breaking buffer (20 mM HEPES-NaOH, pH 7.5, 120 mM NaCl, 2 mM EDTA, 10% glycerol, 100 mM PMSF) and sonicated three times for 1 minute at 35% intensity. After centrifugation at 10,600 g for 20 minutes, Nonidet-P40 (0.5% final concentration) was added and the lysate was incubated with glutathione-Sepharose beads for 30 minutes at 4°C. Beads were washed in lysis buffer (25 mM HEPES-NaOH, pH 7.5, 1% Nonidet-P40, 10 mM MgCl₂, 100 mM NaCl, 5% glycerol, 100 mM PMSF, phosphatase inhibitors) and in binding buffer (25 mM HEPES-NaOH, pH 7.5, 0.5% Nonidet-P40, 30 mM MgCl₂, 40 mM NaCl, 1 mM dithiothreitol). HEK293 cells were transfected with the indicated constructs and scraped with ice-cold lysis buffer. Lysates were centrifuged for 30 seconds at 20,800 g, the supernatant incubated with 20 µg GST-CRIB beads for 30 minutes at 4°C and washed with binding buffer. Finally, beads were resuspended in 20 µl sample buffer and half resolved by SDS-PAGE with 15 µl of lysate to determine the total amount of Rac1 per sample. Nitrocellulose membranes were probed using an anti-Rac1 antibody.

For the RhoG activation assay, a plasmid encoding the GST-tagged RhoG-binding domain of kinectin (KID1) (Vignal et al., 2001) was transformed into *E. coli* BL21. Protein expression was induced with 0.1 mM IPTG and bacteria were further incubated at for 3 hours at 25°C, before centrifugation at 6000 g for 15 minutes and resuspension in PBS supplemented with 1 mM DTT, 100 µM PMSF and 1 µg/ml lysozyme. The lysate was incubated for 30 minutes on ice and subsequently 0.1% Triton X-100, 10 mM MgCl₂ and 0.1 µg/ml DNase (final concentrations) were added. After 30 min on ice, the lysate was centrifuged for 25 minutes at 138,000 g. The supernatant was incubated with glutathione-Sepharose beads pre-equilibrated in PBS, 10 mM DTT, 1% Triton X-100. Pellets were washed with PBS, 10 mM DTT, 1% Triton X-100 and at least 20 µg GST-KID1 was subjected to GST-pull-down per condition. The pull-down was performed as described for the Rac1 activation assay using a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT and protease inhibitors, and a binding buffer containing 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 5 mM MgCl₂, 1% Triton X-100 and protease inhibitors. Finally, beads were resuspended in 20 µl SDS sample buffer and separated by SDS-PAGE together with 15 µl of the lysate to determine the total RhoG amount per sample. PVDF membranes were probed with an anti-RhoG antibody.

Membrane cytosol fractionation

HEK293 cells transfected with the indicated constructs were scraped into homogenisation buffer (20 mM HEPES-KOH, pH 7.2, 2 mM EDTA, 150 mM KCl, supplemented with Roche Complete protease and phosphatase inhibitors), then lysed by passing the cells through a 27G needle. Cell lysates were centrifuged at 3500 g for 5 minutes at 4°C to pellet unbroken cells and nuclei. The postnuclear supernatant was centrifuged at 100,000 g (45,000 rpm, TLA45 rotor) for 1 hour at 4°C. Membrane pellets were resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5% Triton X-100, 1% deoxycholate, 0.1% SDS). Finally, 1/50th of membrane and cytosol fractions were resolved by SDS-PAGE. PVDF membranes were probed with anti-syntaxin6 antibodies as a marker for the membrane fraction and with anti-SOD1 antibodies for the cytosol.

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