

# APP Anterograde Transport Requires Rab3A GTPase Activity for Assembly of the Transport Vesicle

Anita Szodorai,<sup>2</sup> Yung-Hui Kuan,<sup>2,9</sup> Silke Hunzelmann,<sup>1,2</sup> Ulrike Engel,<sup>4</sup> Ayuko Sakane,<sup>6</sup> Takuya Sasaki,<sup>6</sup> Yoshimi Takai,<sup>7</sup> Joachim Kirsch,<sup>3</sup> Ulrike Müller,<sup>5</sup> Konrad Beyreuther,<sup>2,10</sup> Scott Brady,<sup>8</sup> Gerardo Morfini,<sup>8</sup> and Stefan Kins<sup>1,2</sup>

<sup>1</sup>Technical University of Kaiserslautern, Department of Human Biology and Human Genetics, D-67663 Kaiserslautern, Germany, <sup>2</sup>Centre for Molecular Biology, <sup>3</sup>Department of Anatomy and Cell Biology, <sup>4</sup>Nikon Imaging Center, Bioquant, and <sup>5</sup>Institute for Pharmacia and Molecular Biotechnology, University of Heidelberg, D-69120 Heidelberg, Germany, <sup>6</sup>Department of Biochemistry, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan, <sup>7</sup>Division of Molecular and Cellular Biology and Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine/Faculty of Medicine, Kobe 650-0017, Japan, <sup>8</sup>Department of Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, Illinois 60612, <sup>9</sup>Max Planck Institute for Brain Research, D-60528 Frankfurt, Germany, and <sup>10</sup>Network Aging Research, D-69115 Heidelberg, Germany

The amyloid precursor protein (APP) is anterogradely transported by conventional kinesin in a distinct transport vesicle, but both the biochemical composition of such a vesicle and the specific kinesin-1 motor responsible for transport are poorly defined. APP may be sequentially cleaved by  $\beta$ - and  $\gamma$ -secretases leading to accumulation of  $\beta$ -amyloid (A $\beta$ ) peptides in brains of Alzheimer's disease patients, whereas cleavage of APP by  $\alpha$ -secretases prevents A $\beta$  generation. Here, we demonstrate by time-lapse analysis and immunisolations that APP is a cargo of a vesicle containing the kinesin heavy chain isoform kinesin-1C, the small GTPase Rab3A, and a specific subset of presynaptic protein components. Moreover, we report that assembly of kinesin-1C and APP in this vesicle type requires Rab3A GTPase activity. Finally, we show cleavage of APP in transport vesicles by  $\alpha$ -secretase activity, likely mediated by ADAM10. Together, these data indicate that maturation of APP transport vesicles, including recruitment of conventional kinesin, requires Rab3 GTPase activity.

## Introduction

The amyloid precursor protein (APP) is a type I transmembrane protein undergoing processing through sequential cleavage by either  $\alpha$ -secretase, including ADAM10 or ADAM17 (ADAM: a disintegrin and metalloproteinase), or  $\beta$ -secretase BACE1 ( $\beta$ -site APP cleaving enzyme 1) and a proteolytic complex termed  $\gamma$ -secretase (Selkoe, 2001; Reinhard et al., 2005).  $\beta$ -Amyloid (A $\beta$ ) peptides, which accumulate in brains of Alzheimer's disease patients, are produced by the sequential activities of BACE1 and  $\gamma$ -secretase. Initial studies speculated that APP is rapidly transported in a membranous axonal organelle that also contains BACE1 and  $\gamma$ -secretase and that these proteases promoted APP proteolysis within transport vesicles during transit along the axon (Kamal et al., 2001). In contrast, others reported that Presenilin1 (PS1) and BACE1 have transport kinetics distinct from those of APP (Lazarov et al., 2005; Goldsbury et al., 2006).

Anterograde transport of APP is clearly mediated by conventional kinesin (kinesin, kinesin-1) (Koo et al., 1990; Ferreira et al.,

1992; Amaratunga et al., 1993; Simons et al., 1995; Tienari et al., 1996) with velocities of up to 10  $\mu\text{m/s}$  (Kaether et al., 2000; Stamer et al., 2002; Goldsbury et al., 2006) in a vesicle type morphologically distinct from that of synaptophysin-containing vesicles (Kaether et al., 2000). However, the precise mode of connecting conventional kinesin with APP-containing transport vesicles remains controversial, and various models have been proposed. Based on immunoprecipitation experiments (Kamal et al., 2000) and fast axonal transport (FAT) studies in squid axoplasm (Satpute-Krishnan et al., 2006), a direct interaction of the APP C terminus with kinesin light chains (KLCs) was proposed. Inomata et al. (2003) later reported that the interaction between APP and KLCs might be mediated by c-Jun N-terminal kinase-interacting protein 1b (JIP1b). However, more recent data showed that knockdown of JIP1b did not affect localization of APP at the distal end of neurites (Kins et al., 2006) and that KLCs do not interact directly with the cytoplasmic tail of APP (Lazarov et al., 2005), arguing against an essential role of the APP C terminus and JIP1b in the anterograde transport of APP. Moreover, heterologously expressed APP lacking the C-terminal intracellular domain continued to be transported anterogradely along the axon, indicating that the C terminus of APP was not required for anterograde FAT (Tienari et al., 1996; Torroja et al., 1999; Back et al., 2007; Rusu et al., 2007). Together, these data argue that APP does not interact directly with conventional kinesin but do not indicate how conventional kinesin mediates anterograde APP transport. Using biochemical and live cell imaging approaches, we show unequivocally that the APP C terminus is not essential for anterograde FAT of APP. Moreover, our data indicate that Rab3A GTPase activity is required for assembly of

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Correspondence should be addressed to Stefan Kins, Technical University of Kaiserslautern, Department of Human Biology and Human Genetics, Erwin-Schrödinger-Straße 13, D-67663 Kaiserslautern, Germany. E-mail: s.kins@biologie.uni-kl.de.

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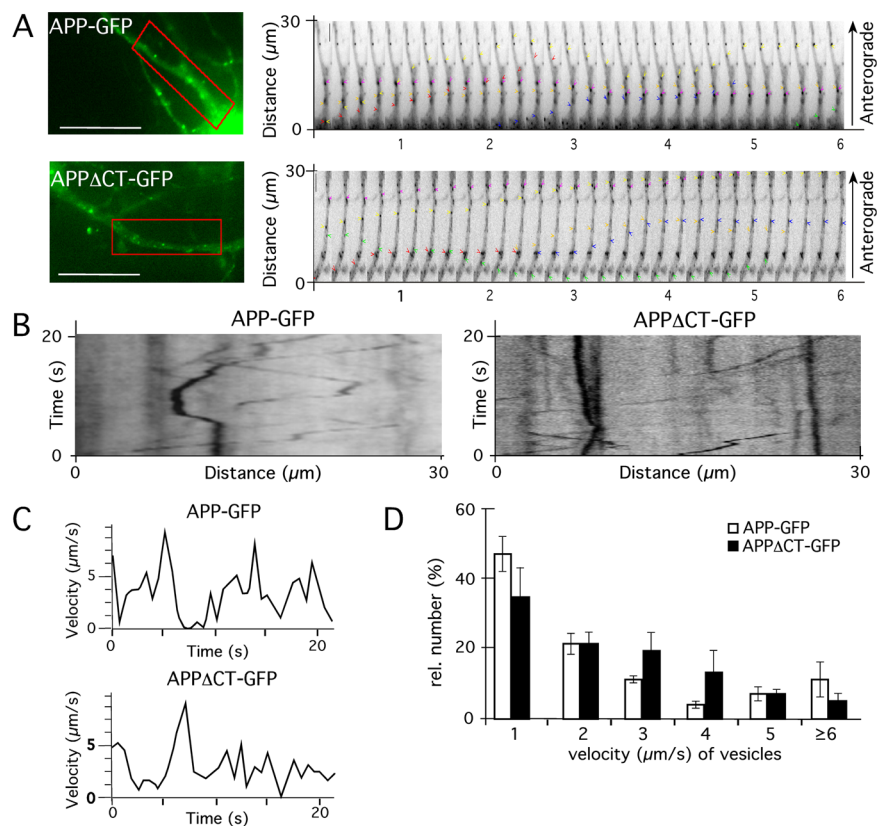


tive, FITC or cyanine 5 filters) as described in detail previously (Kuan et al., 2006).

## Results

### Anterograde fast axonal transport of APP is independent of its intracellular domain

Immunocytochemical and immunohistochemical analyses of APP lacking the C terminus (APP $\Delta$ C) in primary neurons and *Drosophila* motor neurons revealed that APP undergoes anterograde FAT in the absence of its C terminus (Tienari et al., 1996; Torroja et al., 1999; Back et al., 2007; Rusu et al., 2007). However, these experiments did not examine whether APP anterograde FAT rates might be altered by deletion of the APP C-terminus, nor did they evaluate whether APP and APP $\Delta$ C are cotransported in the same type of vesicles. To address these issues, we performed live microscopy studies of GFP fusion proteins with APP (APP-GFP) and APP lacking the C terminus (APP $\Delta$ C-GFP) in primary neurons. Mixed cortical neurons (DIV7) were transfected with cDNAs encoding either APP-GFP or APP $\Delta$ C-GFP and analyzed by time-lapse microscopy 18 h after transfection (Fig. 1A–C). Velocity analysis revealed that APP-GFP is transported with a maximal velocity of  $\sim 7$ – $10$   $\mu\text{m/s}$  (Fig. 1) (supplemental Fig. 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), consistent with previous studies (Kaether et al., 2000; Goldsbury et al., 2006). Detailed analyses of APP-GFP and APP $\Delta$ C-GFP transport rates (Fig. 1D) revealed that APP $\Delta$ C-GFP movement was indistinguishable from full-length APP-GFP, arguing that the APP C terminus is not required for packaging of APP in the anterograde transport vesicles or docking of conventional kinesin. To determine whether APP and APP $\Delta$ C are transported in the same type of vesicles, we cotransfected primary neurons with APP-RFP and APP $\Delta$ C-GFP. The two fluorescent proteins were visualized sequentially with a time interval of 200 ms (exposure time and time for changing the filter). We observed that anterograde movements of both fusion proteins were highly correlated. After tracking anterogradely transported vesicles, velocity kinetics were determined. This analysis revealed identical trafficking characteristics for APP-RFP and APP $\Delta$ C-GFP (supplemental Fig. 1B, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), indicating that they were cotransported in the same type of vesicle. Consistent with previous studies (Kaether et al., 2000), we found that synaptophysin-CFP was transported in a different type of transport vesicle from APP-RFP and APP $\Delta$ C-GFP (data not shown), indicating that heterologously expressed APP fusion proteins are targeted to a specific type of transport vesicles. Recently, we reported that APP forms homotypic *cis*-dimers (Soba et al., 2005). To exclude the possibility that APP $\Delta$ C-GFP might be cotransported by associating to endogenous APP, we used primary neurons from APP knock-out mice transfected with APP $\Delta$ C-GFP for time-lapse analysis. These

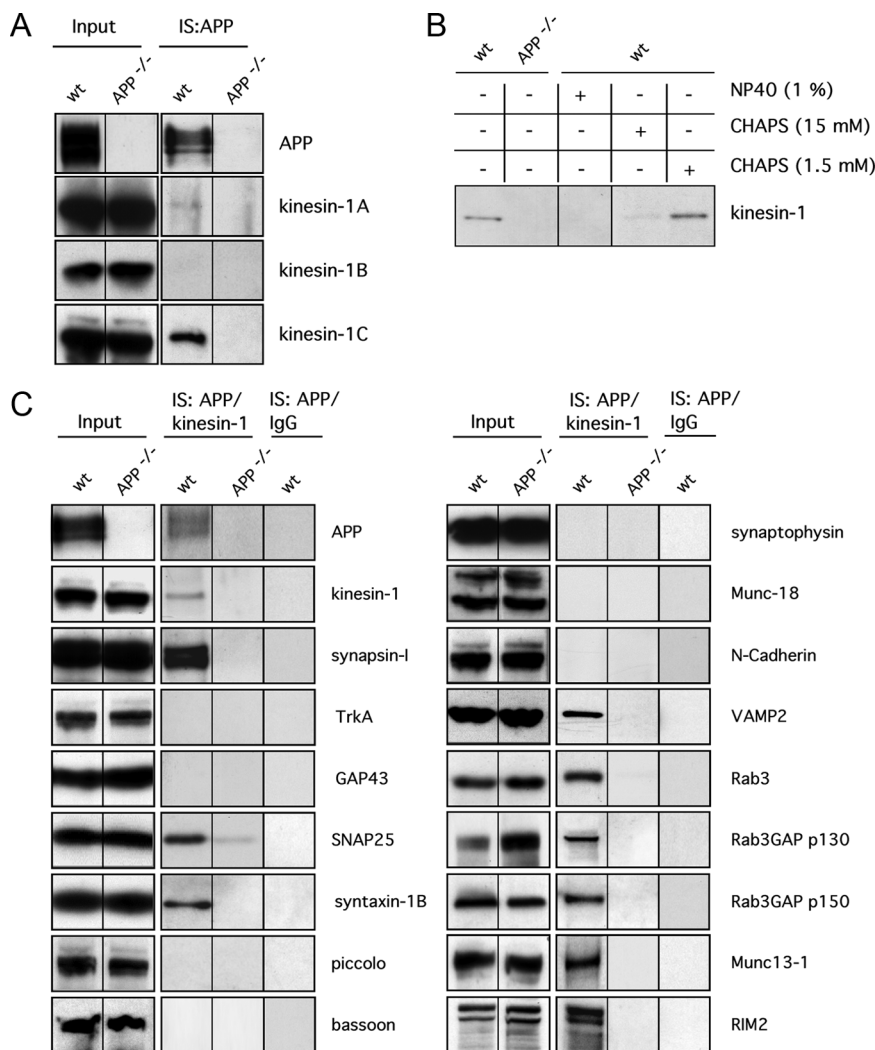


**Figure 1.** APP can be transported by the fast axonal transport machinery in the absence of its intracellular C terminus. Mouse primary cortical neurons (DIV7) expressing APP-GFP and/or APP $\Delta$ C-GFP were analyzed by time-lapse microscopy 18 h after transfection. **A**, Fluorescence micrographs of APP-GFP- and APP $\Delta$ C-GFP-expressing neurons. For tracking and velocity analyses, a region of interest (red box) was selected. Sequential images of the region of interest are shown to the right. Colored arrowheads indicate the single vesicles that have been examined. Time interval between images was 200 ms, 5 images/s. **B**, Representative kymographs showing APP-GFP and APP $\Delta$ C-GFP movement. **C**, Velocities of APP-GFP- and APP $\Delta$ C-GFP-containing vesicles, assayed over a period of 22 s. **D**, Histogram showing quantification of the number of recorded vesicles moving at velocities of 1, 2, 3, 4, 5, or  $>6$   $\mu\text{m/s}$ . No statistical significant differences between the velocities of APP-GFP- and APP $\Delta$ C-containing transport vesicles could be determined (Student's *t* test,  $p \geq 0.2$ ). Scale bar, 25  $\mu\text{m}$ .

studies showed that the FAT machinery transports APP $\Delta$ C-GFP even in the absence of endogenous APP (supplemental Fig. 1A, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Consistently, analysis of APP-GFP and APP $\Delta$ C-GFP transport in APP/APLP1/APLP2 triple-knock-out mouse embryonic fibroblasts revealed no differences of their fast transport characteristics (1–4  $\mu\text{m/s}$ ) (data not shown). Together, these data suggest that the APP C terminus is not required for FAT of APP.

### APP is a cargo of a kinesin-1C associated transport vesicle

Previous studies indicated that APP-containing vesicles are transported by conventional kinesin. The holoenzyme of conventional kinesin exists as a tetramer consisting of two KLCs and two kinesin heavy chain (kinesin-1, KHC, KIF5s) subunits (DeBoer et al., 2008). Following the standard nomenclature for kinesins, the term “conventional kinesin” herein refers to the tetrameric motor protein complex (heavy and light chains), whereas “kinesin-1” refers exclusively to the heavy chain subunits (Lawrence et al., 2004; DeBoer et al., 2008). To identify specific kinesin-1 isoform(s) linked to APP-containing vesicles and characterize the APP cargo vesicle, we performed immunoprecipitations from crude mouse brain membrane fractions, using magnetic beads loaded with an antibody directed against the C terminus of APP (CT20-coupled beads). APP immunoprecipitates were analyzed by



**Figure 2.** APP is a cargo of an axonal transport vesicle containing presynaptic components and is associated with kinesin-1C. **A**, Immunoprecipitations (IS) of APP using an anti-APP antibody (CT20) from wild-type (wt) and APP knock-out (APP<sup>-/-</sup>) mouse brain homogenates. Each lysate (1/100) was loaded as input control (Input). Western blot analyses of APP immunoprecipitates with antibodies against specific kinesin-1s revealed that APP is mainly associated with kinesin-1C. **B**, Immunoprecipitated APP-containing membrane preparations from wild-type mice (wt) were treated with 1% (v/v) NP-40 (lane 3), 15 mM CHAPS (lane 4), 1.5 mM CHAPS (below the critical micellar concentration) (lane 5), or no detergent (lane 1). An immunoprecipitate using anti-APP antibody obtained from APP knock-out mice (APP<sup>-/-</sup>) is shown as a control (lane 2). **C**, Total mouse brain membrane fractions were separated on a linear iodixanol gradient (supplemental Fig. 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), and low-density membrane fractions (fractions 1–5) were pooled and used for a two-step immunoprecipitation: vesicles were first immunoprecipitated with an anti-APP antibody (IS:APP). After these, immunoprecipitated membranes were eluted and subject to immunoprecipitation with an anti-kinesin-1 antibody (H2) (IS: APP/kinesin-1) (for details, see Materials and Methods). Coimmunoprecipitated proteins were separated by SDS-PAGE and subjected to Western blot analysis. Brain homogenates from APP knock-out (APP<sup>-/-</sup>) mice were used for immunoprecipitation with anti-APP antibody as a control for nonspecific binding. To test for specificity of anti-kinesin-1 immunoprecipitations, a non-immune IgG fraction (IgG) was used (IS:APP/IgG).

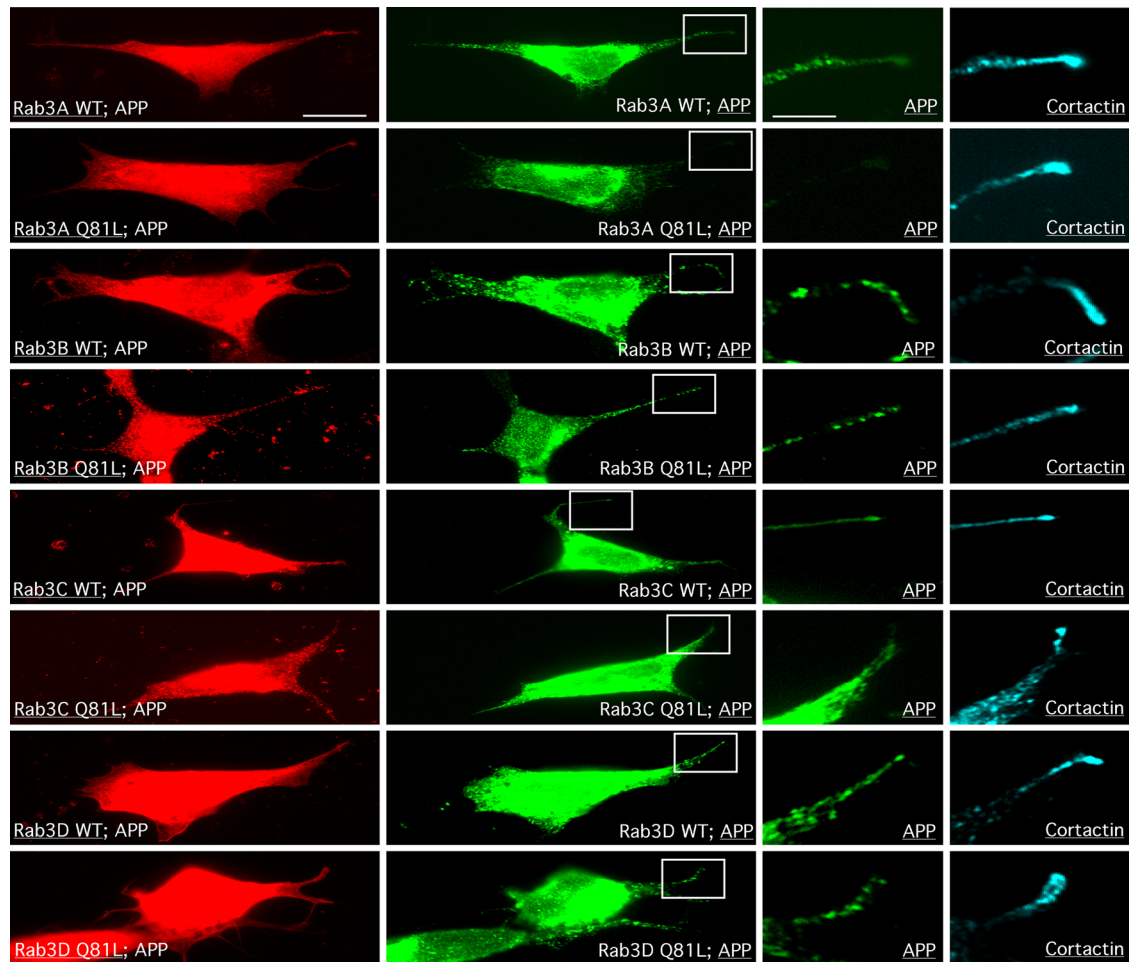
immunoblotting using antibodies that specifically recognize different kinesin-1 gene products: kinesin-1A, kinesin-1B, and kinesin-1C [formerly KIF5A, KIF5B, and KIF5C, respectively (Lawrence et al., 2004)] (DeBoer et al., 2008) (Fig. 2A). No immunoreactivity was detected with an antibody against kinesin-1B (specific antibodies used are described in Materials and Methods), and barely detectable amounts of kinesin-1A were present in the APP immunoprecipitates. The anti-kinesin-1 antibody (H2) reacting with both kinesin-1A and kinesin-1C (DeBoer et al., 2008) displayed significant immunoreactivity when reacted with APP immunoprecipitated fractions (Fig. 2A). The H2 immunoreactive band in the APP immunoprecipitates migrated at a significant lower molecular weight than kinesin-1A and

could therefore be clearly identified as kinesin-1C (DeBoer et al., 2008). Thus, conventional kinesin associated with APP-containing membranes is mainly composed of kinesin-1C. Specificity of the kinesin-1C immunoreactivity in APP immunoprecipitates was verified using brain homogenates from APP knock-out mice (Fig. 2A). In the absence of endogenous APP, we did not observe nonspecific binding of any kinesin-1 isoform to immunoprecipitates obtained with the CT20-loaded magnetic beads (Fig. 2A).

APP can be associated with a variety of membrane structures, including Golgi, endoplasmic reticulum (ER), and transport vesicles (Kins et al., 2006). To enrich for membrane fractions containing axonal transport vesicles, we separated the brain extracts in a linear iodixanol gradient (5–23%). We thus obtained low-density membrane material enriched with presynaptic marker proteins such as GAP43, synapsin-1A/B, and synaptophysin (supplemental Fig. 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). These membranes were mostly separated from ER or *cis*-Golgi membrane fractions, as evidenced by low levels of typical ER and *cis*-Golgi markers (anti-Grp78 and anti-KDEL). Significantly, APP immunoprecipitates obtained from pooled membrane fractions 1–5 from wild-type mouse brains also contained kinesin-1C (Fig. 2B). As before (Fig. 2A), immunoprecipitates prepared from APP knock-out mouse brain contained no kinesin-1 immunoreactivity and served as negative controls (Fig. 2B). Treatment of the APP-immunoprecipitated membranes with detergents (i.e., 1% NP-40 or 15 mM CHAPS) caused a loss of kinesin-1C from APP immunoprecipitates (Fig. 2B). In contrast, treatment of immunoprecipitates with concentrations of CHAPS (1.5 mM) below the critical micellar concentration (Kratohvil, 1984; Partearroyo et al., 1988) did not cause a dissociation of kinesin-1C from APP beads (Fig. 2B). Crosslinking of APP immunoprecipitates with DMP before treatment with detergent did not maintain the APP/kinesin interaction (data not shown). Combined with the live cell analyses of transport of APP lacking the C terminus, these data suggest that conventional kinesin containing kinesin-1C heavy chains associates with APP transport vesicles in a manner independent of the APP C terminus.

### Characterization of APP transport vesicles

To characterize APP-containing membrane compartments moving in FAT (i.e., transport vesicles) in more detail, we performed two sequential immunoprecipitations with CT20 and H2-loaded magnetic beads. APP-containing membrane compartments were prepared as described previously using beads linked with a disul-



**Figure 3.** Rab3-subtype-specific influence on APP localization. To determine specific Rab3 family members that might be essential for anterograde transport of APP, we tested the influence of both wild-type (wt) and GTPase-deficient mutant (Q81L) versions of GFP-tagged Rab3A–Rab3D on APP-HA localization at the distal end of growing neurites. Neuroblastoma cells (SH-SY5Y) were cotransfected with the different Rab3 constructs together with APP-HA cDNA and analyzed by immunocytochemistry using anti-GFP (red), anti-HA (green), and as a growth cone marker anti-cortactin (cyan) antibodies. Notably, only transfection with the Rab3A Q81L construct caused a reduction in the level of APP that normally accumulates at the distal ends of neurites. To visualize the neurites more clearly, the regions of interest (boxed) are shown at higher magnification in the two right columns. Scale bar, 10  $\mu\text{m}$ . Higher-magnification bar, 3.3  $\mu\text{m}$ .

fide crosslinker to CT20 antibodies. CT20 immunisolates were then treated with DTT to elute APP-containing membranes and subjected to a second round of immunoisolation using H2-loaded magnetic beads, allowing for the enrichment of membranes containing both APP and kinesin-1C.

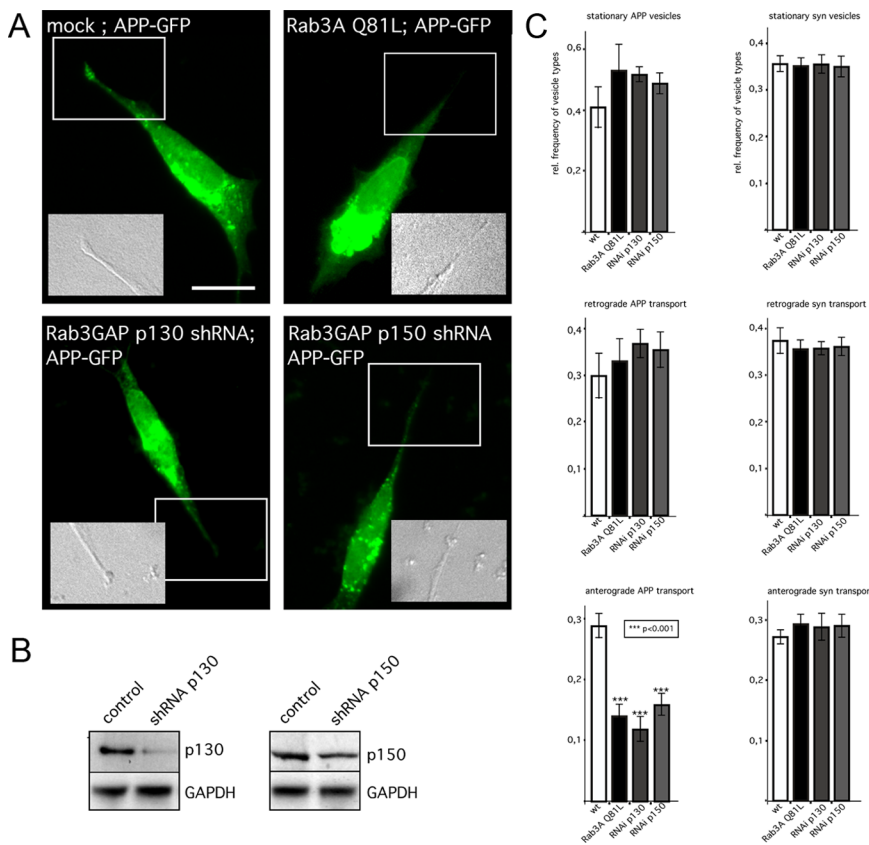
Double APP/kinesin-1-immunisolated membrane preparations were subjected to Western blot analysis, using various antibodies directed against a wide range of presynaptic and postsynaptic protein components. Only a subset of presynaptic proteins was detected in these APP/kinesin-1C double immunisolates, including SNARE complex components (such as VAMP2, SNAP25, and syntaxin-1b) and synapsin-I, as well as components of the active zone, such as Rab3, RIM2, Munc13-1, and Rab3GAP p130 and p150 (Fig. 2C). Other presynaptic components previously described as putative cargoes of APP transport vesicles, including GAP43 and TrkA (Kamal et al., 2001), were not found in our membrane immunisolates. Synaptophysin, a presynaptic protein previously shown to be transported in a different class of membranous organelles than APP (Kaether et al., 2000), was also absent. Neither postsynaptic proteins, such as the NMDA or AMPA receptor (data not shown), nor presynaptic cytomatrix proteins (Dresbach et al., 2001), such as bassoon and

piccolo were detected in APP/kinesin-1C double immunisolates (Fig. 2C). To control for specificity of the double immunisolates, we used membrane preparations from APP knock-out mouse brain in the first APP immunoisolation step. Specificity of the kinesin-1 immunoisolation was verified by using non-immune mouse Ig (Fig. 2C).

Immunoisolation experiments here show that APP/kinesin-1 coimmunisolated membrane fractions contain a subset of presynaptic protein contents that included syntaxin, synapsin-I, Rab3, and several Rab3A associated proteins but did not include synaptophysin or typical protein components of the active zone, such as bassoon or piccolo. Together, these data strongly suggest that APP and other presynaptic membrane protein represent cargoes present in a biochemically distinct presynaptic membranous organelle, which in turn associates primarily with kinesin-1C.

#### APP localization at growth cones depends on Rab3A GTPase activity

Interestingly, some of the components identified in APP transport vesicles (i.e., Rab3GAP p130, Rab3GAP p150) are known to interact with and to stimulate the GTPase activity of Rab3 family members (Rab3A–Rab3D) (Fukui et al., 1997; Sakane et al.,



**Figure 4.** Rab3 GTPase activity is essential for APP fast anterograde transport. Neuroblastoma cells (SH-SY5Y) were cotransfected with APP–GFP cDNA and empty vector (mock), Rab3A Q81L cDNA, shRNA constructs directed against Rab3GAP p130, or Rab3GAP p150. **A**, Mock-transfected cells expressing APP–GFP displayed an accumulation of APP–GFP at the tips of neurites (box), whereas inhibition of Rab3A GTPase activity by expression of mutant Rab3A Q81L or silencing of Rab3GAP p130 or p150 caused a decrease of APP–GFP levels at the tips. Differential interference contrast images of the regions of interest/of the indicated regions (boxed) are shown as insets. Scale bar, 10  $\mu\text{m}$ . **B**, The silencing efficiency of the human Rab3GAP p130 or Rab3GAP p150 shRNA was of  $\sim 40$ –60%. Taking into account that the transfection rate with the vector-based shRNA constructs was 50–60%, the knockdown efficiency per transfected cell was estimated to be  $\sim 70$ –80%. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. **C**, APP–GFP (left) or synaptophysin–GFP (right) fusion proteins were analyzed by time-lapse microscopy (5 frames/s) in mock-transfected neuroblastoma cells (white columns), cells coexpressing Rab3A Q81L (black columns), or cells with knockdown [RNA interference (RNAi)] of Rab3GAP p130 (dark gray columns) or Rab3GAP p150 (light gray columns). Kymographs from single cells ( $n = 12$ ) were analyzed. The relative frequencies of stationary, retrogradely, or anterogradely transported APP–GFP (left) or synaptophysin–GFP (right) containing vesicles was determined. The range of velocities for synaptophysin–GFP (syn) is lower (1–5  $\mu\text{m/s}$ ) than the range seen with APP (1–10  $\mu\text{m/s}$ ). Therefore, we determined only the relative frequency of anterograde APP–GFP vesicles moving at velocities  $\geq 4 \mu\text{m}$ . Error bars represent SEM. \*\*\* $p < 0.001$ ,  $t$  test.

2006). APP typically accumulates at the distal end of neurites (Ferreira et al., 1993; Morfini et al., 1997; Sabo et al., 2003), and inhibition of anterograde APP transport affects this accumulation (Ferreira et al., 1992; Morfini et al., 1997). We cotransfected neuroblastoma SH-SY5Y cells with APP–HA and specific Rab3 isoforms (Rab3A–Rab3D) and then examined accumulation of APP–HA at the growth cones of neurites (identified by costaining with the growth cone marker cortactin). Rab3 constructs used in these experiments included wild-type forms or GTPase-deficient mutants carrying a Q81L amino acid replacement (Johannes et al., 1994) (kind gifts from M. Zerial) (Fig. 3B). Significantly, only coexpression of the Rab3A GTPase-deficient mutant caused a reduction of APP levels at the neurite ends, suggestive of a deficit in the anterograde transport of APP. None of the other wild-type or mutant Rab3 isoforms altered APP distributions in SH-SY5Y cells (Fig. 3). These findings suggested that Rab3A GTPase activity is required for anterograde FAT of APP.

### Rab3A GTPase activity is essential for fast anterograde transport of APP

As an independent test of the hypothesis that Rab3A GTPase activity is required for APP anterograde transport, we knocked down Rab3GAP p130 and Rab3GAP p150 in SH-SY5Y cells using shRNA constructs. Western blot analysis showed a 40–60% reduction in Rab3GAP p130 levels (Fig. 4B). Because the transfection efficiency with the vector-based shRNA constructs was 50–60%, the knockdown efficiency per transfected cell was estimated to be  $\sim 70$ –80%. Rab3GAP knockdown cells expressing APP–GFP exhibited accumulation of APP in the cell soma, and APP–GFP failed to accumulate in neurite ends (Fig. 4A). In contrast, mock-transfected cells exhibited the typical APP–GFP accumulations at neurite ends (Fig. 4A). Notably, overexpression of Rab3GAPs p130 and p150 together with APP led to a more pronounced accumulation of APP at the growing tips (supplemental Fig. 3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). To characterize the role of Rab3A GTPase activity in anterograde transport of APP further, we transfected SH-SY5Y cells with either the Rab3A Q81L mutant construct or with shRNA constructs directed against Rab3GAP p130 and p150. Cells were analyzed by time-lapse microscopy (5 frames/s) 18 h after APP–GFP cDNA transfection. APP–GFP-expressing cells cotransfected with empty vector (Fig. 4C, white column) or wild-type Rab3A coexpressing cells served as controls (data not shown). To evaluate changes in APP transport rates, we calculated the relative frequencies of stationary ( $\leq 0.2 \mu\text{m/s}$ ), retrogradely ( $> 0.2 \mu\text{m/s}$ ), and anterogradely ( $> 0.2 \mu\text{m/s}$ ) transported APP–GFP-containing vesicles in 12 or more individual cotransfected cells for each construct tested. In neurites of control vector-transfected cells, we found

in distal regions  $41 \pm 7\%$  (mean  $\pm$  SEM) stationary,  $30 \pm 5\%$  (mean  $\pm$  SEM) retrogradely, and  $29 \pm 2\%$  (mean  $\pm$  SEM) anterogradely transported APP–GFP vesicles (Fig. 4C). The relative frequencies of anterograde transport vesicles were significantly reduced after inhibition of Rab3A GTPase activity with the Rab3A Q81L mutant (14%) or silencing of Rab3GAP p130 (12%) or Rab3GAP p150 (16%) ( $t$  test,  $p < 0.001$ ) (Fig. 4C), but frequencies of retrogradely transported and stationary membrane compartments were not significantly altered (Fig. 4C). These data indicate that Rab3A GTPase activity modulated fast anterograde transport of APP. To make sure that inhibition of Rab3A GTPase does not cause a general block of anterograde FAT, we evaluated FAT of synaptophysin-containing vesicles, which is carried in a different cargo vesicle than APP (Kaether et al., 2000) (Fig. 4C). Neither mutant Rab3A Q81L nor silencing of Rab3GAP p130 or p150 affected the relative frequencies of synaptophysin–GFP vesicles found

in anterograde or retrograde FAT, suggesting that inhibition of Rab3A GTPase specifically affects the anterograde FAT of APP-containing vesicles. Together, these findings suggest that Rab3A GTPase activity is required for anterograde FAT of APP.

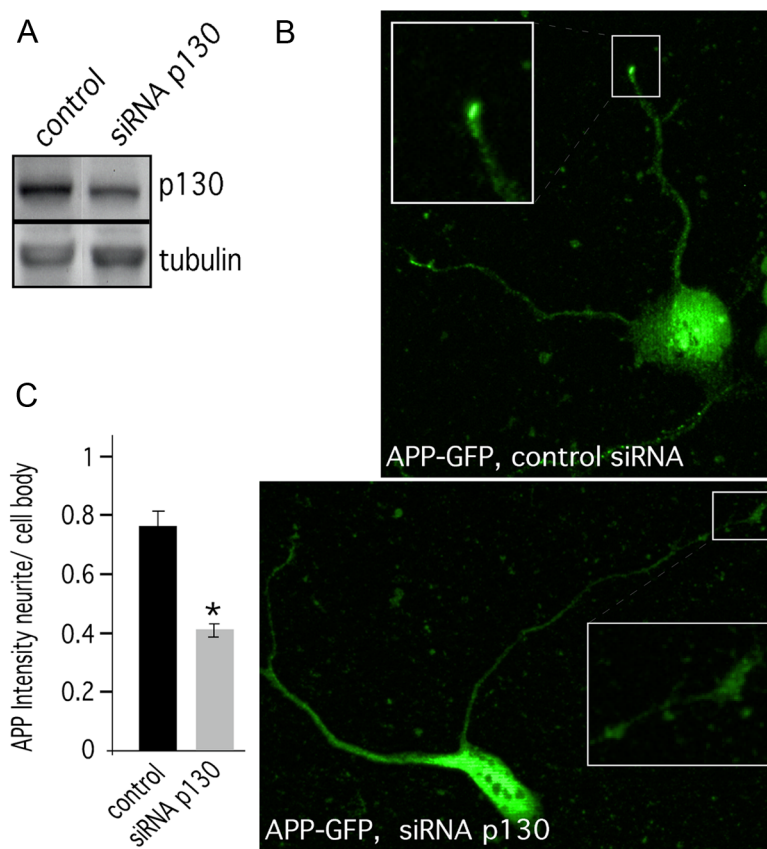
We also investigated the effects of Rab3GAP p130 knockdown on APP localization using primary cultured neurons. We treated stage 1 primary neurons with Rab3GAP p130 siRNA and analyzed the localization of endogenous APP (Fig. 5*B*). Treatment with Rab3GAP p130 siRNA caused a statistical significant reduction of Rab3GAP p130 levels to  $64 \pm 9\%$ . As observed in SH-SY5Y cells, a clear reduction of APP accumulation at the tips of neurites was seen in primary neurons treated with the Rab3GAP p130 siRNA construct compared with control transfected cells (Fig. 5*B,C*). For quantification, we measured the intensity of GFP-APP in the cell body and the tip of neurites of single cells treated with control or Rab3GAP p130 siRNA ( $n = 27$  for each) (Fig. 5*C*). Consistent with data obtained from SH-SY5Y cells, the ratio of GFP-APP intensity at tips/cell bodies was reduced by  $\sim 40\%$  in Rab3GAP p130 siRNA-treated cells compared with control treated cells. Silencing of Rab3GAP p130 caused a reduction of APP intensity at the tips and an accumulation of APP in the cell bodies, suggesting that Rab3A GTPase activity is also required for anterograde FAT of APP in primary neurons.

#### The activities of Rab3GAP p130 and p150 are required for the attachment of conventional kinesin to APP-containing vesicles

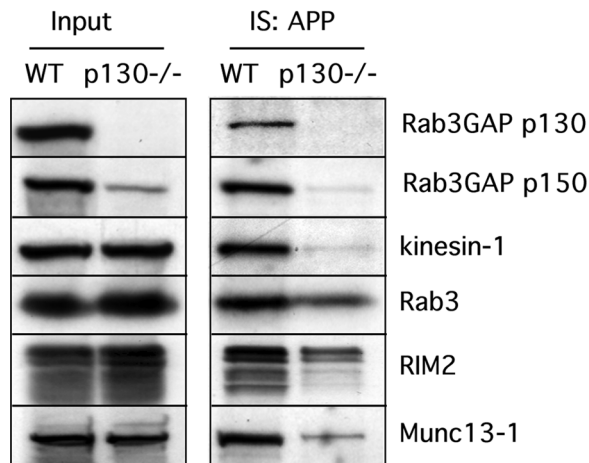
Given that Rab3 GTPase activity is required for the anterograde FAT of APP, we speculated that conversion of Rab3A from GTP- to-GDP bound states might be crucial for the correct assembly of APP transport vesicles. To test this possibility, we used isolated membranes from Rab3GAP p130 knock-out mice, which display reduced levels of Rab3A activity (Sakane et al., 2006). Significantly, the amounts of kinesin-1, Rab3, and the Rab3 associated proteins RIM2 and Munc13-1 were dramatically decreased in APP immunisolates derived from Rab3GAP p130 knock-out brains compared with immunisolates derived from wild-type mouse brain (Fig. 6). Regarding the activity-dependent association of Rab3A with membranes, this appears consistent with the reduction in Rab3A activity reported in brains of p130<sup>-/-</sup> knock-out mice (Sakane et al., 2006). Together, these data indicate that Rab3A GTPase activity is required for the correct assembly of APP into anterograde transport vesicles, including the packaging of membrane proteins, such as Rab3A, and the association of the motor protein conventional kinesin.

#### APP and ADAM10 are cargoes of a common transport vesicle

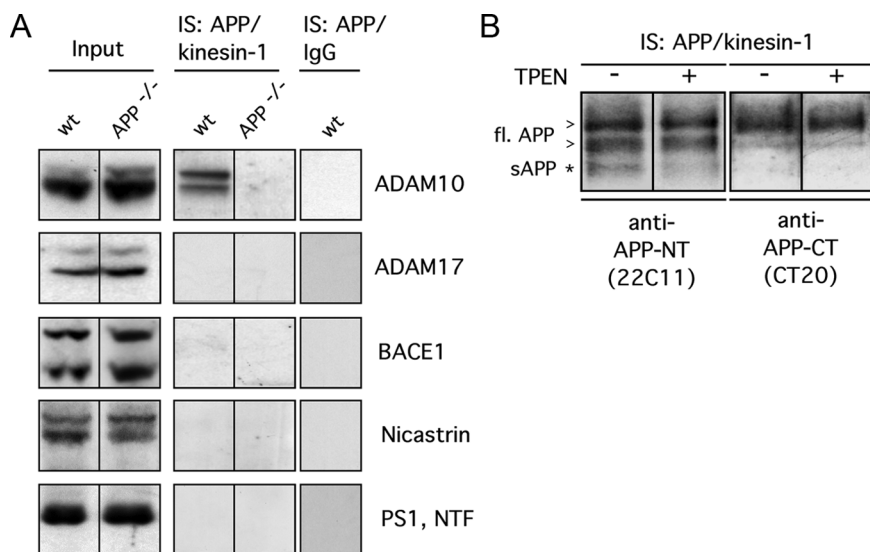
In this study, we identified several novel putative cargo proteins present in APP-containing transport vesicles. The question of



**Figure 5.** Inhibition of Rab3 GTPase activity reduces the levels of APP at the tips of neurites. Mouse primary cortical neurons (DIV1) were transfected with siRNA directed against Rab3GAP p130 or nonfunctional control siRNA. **A**, Western blot analysis indicated that the silencing effect of mouse Rab3GAP p130 was  $\sim 35\%$ . **B**, Immunocytochemistry of the siRNA transfected stage 1 primary neurons using antibody directed against the APP C terminus (CT20) reveals that the cells treated with Rab3GAP p130 siRNA have less APP accumulation at the tips of neurites. For clarity, the tips of the neurites (boxed) are shown at a higher magnification as inset. **C**, For quantification, the intensity of APP in the cell body and in the tip of the neurites was measured using NIH ImageJ. The ratio of APP intensity at tips/cell bodies was reduced  $\sim 40\%$  in cells treated with Rab3GAP p130 siRNA compared with control siRNA transfected cells. Error bars represent SEM. \* $p > 0.01$ , *t* test. Scale bar,  $5 \mu\text{m}$ .



**Figure 6.** Loss of kinesin-1 from APP transport vesicles in Rab3GAP p130 knock-out mice. Low-density membrane fractions from wild-type (wt) or Rab3GAP p130 knock-out mouse (p130<sup>-/-</sup>) brain homogenates were immunisolated with anti-APP antibodies (IS:APP) and subjected to Western blot analyses using antibodies directed against Rab3GAP p130, Rab3GAP p150, kinesin-1, Rab3, and two putative cargoes of APP transport vesicles, RIM2 and Munc13-1 (see Fig. 1). Total lysate ( $1/_{80}$ ) from each brain was loaded in the lanes of the left panel as input control (Input).



**Figure 7.** APP and ADAM10 are cargoes of a common transport vesicle. **A**, Western blot analyses of APP/kinesin-1 double immunisolates (IS:APP/kinesin-1) with anti-ADAM10, anti-ADAM17, anti-BACE1, anti-PS1, and anti-Nicastrin antibodies. As control for APP/kinesin-1 double immunisolations, brain homogenates from APP knock-out mice (APP<sup>-/-</sup>) and non-immune IgGs (IS:IgG) were used. Each lysate (1/80) was loaded as input control (Input). wt, Wild type. **B**, Western blot analyses of APP/kinesin-1 double-immunisolated membrane preparations (IS:APP/kinesin-1) with an antibody directed against the APP N terminus (22C11), recognizing full-length (fl.) APP (arrowhead) and cleaved sAPP (asterisk), as well as an antibody directed against the APP C terminus, recognizing full-length APP (CT20) but not sAPP. TPEN, a zinc chelator blocking  $\alpha$ -secretase activity, was added directly after brain homogenization at a concentration of 10  $\mu$ M when indicated (+).

whether the APP-processing secretases BACE1 and PS1 are also cargo proteins in these vesicles has been controversial (Kamal et al., 2001; Lazarov et al., 2005; Goldsbury et al., 2006). To clarify this issue, we performed APP/kinesin-1 double immunisolations as described above and examined the presence of APP secretases and APP processing in the isolated membrane fractions. In Western blot analysis of APP/kinesin-1 double immunisolates from mouse brain homogenates, neither BACE1 nor PS1 were detectable (Fig. 7A). Nicastrin, another component of  $\gamma$ -secretase, was also undetectable in APP/kinesin-1 double immunisolates (Fig. 7A). We also tested for the presence of the putative  $\alpha$ -secretases ADAM10 and ADAM17. From these, only ADAM10 was detected in double immunisolates obtained from wild-type, but not from APP knock-out, mouse brain, suggesting that APP/kinesin-1 double-immunisolated membrane compartments contain ADAM10 as a cargo protein.

Next, we evaluated APP processing in the isolated APP transport vesicle fraction. For this purpose, we probed APP/kinesin-1 double immunisolates with an antibody directed against the APP N terminus [22C11, which recognizes both full-length APP and cleaved, secreted APP (sAPP)] and with an antibody directed against the APP C terminus (CT20, which recognizes full-length APP but not sAPP) (Fig. 7B). These analyses revealed a clear difference in the detected pattern of APP immunoreactivity. The 22C11 antibody recognized a lower-molecular-weight band, not detected by the C-terminal antibody, representing sAPP. To exclude nonspecific cross-reactivity of the 22C11 antibody, we tested the antibody on brain extracts of wild-type and APP knock-out mice. No immunoreactivity was seen with the 22C11 antibody in APP knock-out mice (data not shown). To test whether APP can be cleaved by  $\alpha$ -secretase in the APP/kinesin-1 double-immunisolated membrane compartment, we treated the double-immunisolated membrane fractions over a time period of  $\sim$ 30 h with a membrane-permeable zinc chelator (10  $\mu$ M TPEN) that inhibits  $\alpha$ -secretase activity (Fonte et al., 2001). The generation of the lower-molecular-weight putative

sAPP band was reduced in the presence of the  $\alpha$ -secretase inhibitor. Together, these data suggest that APP might be processed in the APP/kinesin-1 double-immunisolated membrane compartment by  $\alpha$ -secretase activity, most likely mediated by ADAM10.

## Discussion

### The APP C terminus is not essential for fast anterograde transport

A role of APP as a cargo receptor for conventional kinesin has been a contentious issue (Kamal et al., 2000, 2001; Lazarov et al., 2005). Various reports proposed that transport of APP-containing vesicles depends on a direct interaction between the C terminus of APP and the KLCs of conventional kinesin, suggesting that APP acts as a motor protein adaptor/receptor (Kamal et al., 2000). Contrasting with predictions from this model, we and others showed that APP constructs lacking the C-terminal intracellular domain are transported to the nerve terminal (Tienari et al., 1996; Torroja et al., 1999; Back et al., 2007; Rusu et al., 2007). However, it was unclear whether the anterograde FAT of APP lacking its C terminus was mediated

by mechanisms different from those underlying transport of full-length APP. In this study, time-lapse analyses of fluorescently labeled APP demonstrates that APP lacking its C terminus is packed and transported in the same type of FAT vesicles as full-length APP. Treatment of APP/kinesin-1 coimmunisolated membranes with detergents caused the dissociation of kinesin-1 from the APP-immunisolated membranes, arguing against a direct interaction between these proteins. Together, our data demonstrate that APP is a cargo of a specific subset of membranes transported by conventional kinesin and not a receptor protein directly linking conventional kinesin via its C terminus to this vesicle type. However, additional analyses will be necessary to determine the molecular basis underlying the association of conventional kinesin with APP transport vesicles.

As with many integral membrane proteins, a fraction of the APP is returned by retrograde transport (Lazarov et al., 2007, their Fig. 1). Our analysis of APP-GFP transport included proximal dendrites. Because the microtubule cytoskeleton in this area is not uniformly organized (microtubules may exhibit both polarities), conventional kinesin-dependent transport can take place in both directions (retrogradely and anterogradely relative to the cell body). However, the presence of some APP in the endosomal compartments (Ferreira et al., 1993) is consistent with retrograde FAT.

### Kinesin-1C mediates axonal transport of APP

Based on studies of APP transport after treatment with antisense oligos directed against kinesin-1B, it was assumed that APP anterograde transport is mediated by kinesin-1B (Ferreira et al., 1992; Kaether et al., 2000). However, in these studies, neither a specific reduction of kinesin-1B nor analyses of other kinesin-1 isoforms were performed. Here, we tested systematically for kinesin-1 family members (kinesin-1A, kinesin-1B, kinesin-1C) that might be selectively associated with APP-containing membrane

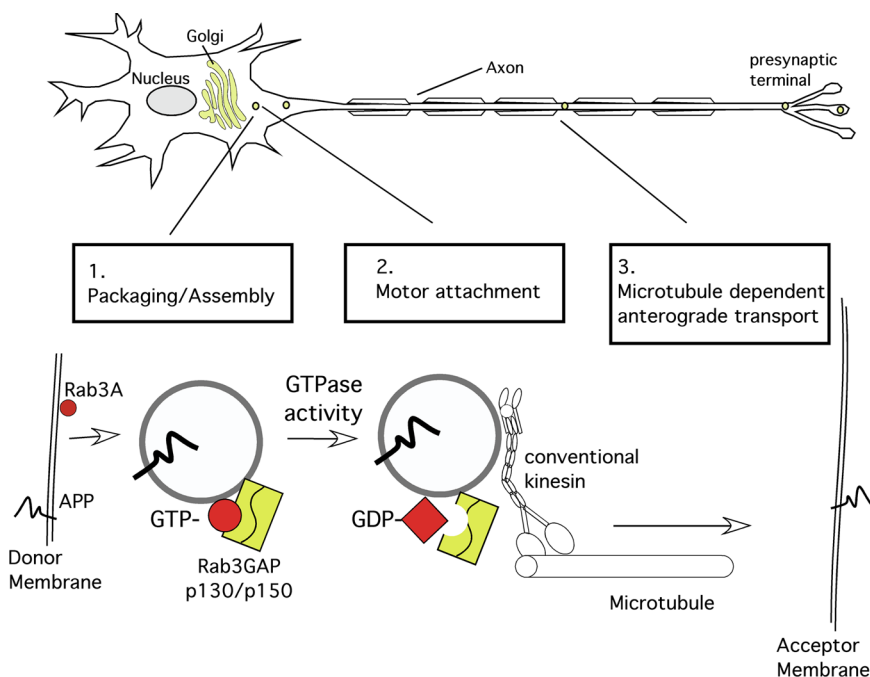


fractions. Interestingly, we found that kinesin-1B was not detectable in APP immunoprecipitates, but kinesin-1C (and to a much lesser extent, kinesin-1A) is preferentially associated with APP transport vesicles. Recently, it was shown that conventional kinesin holoenzymes are formed of kinesin-1 homodimers, which associate with biochemically different cargoes (DeBoer et al., 2008). The selective association of APP-containing transport vesicles with kinesin-1C reported here appears consistent with a role of kinesin-1s in the targeting of conventional kinesin holoenzymes to specific membrane-bounded organelle cargoes (DeBoer et al., 2008).

### A presynaptic transport vesicle subtype contains both Rab3A and APP

Our detailed biochemical characterization of APP transport vesicles in association with kinesin-1C revealed that these membrane compartments contained no postsynaptic protein contents but instead contained a subset of presynaptic proteins, including synapsin-1, SNAP25, syntaxin-1B, VAMP2, Munc13-1, RIM2, Rab3, and Rab3GAP p130 and p150. Significantly, all these protein cargoes are involved in various aspects of synaptic vesicle fusion. Thus, our results here are consistent with previous studies suggesting that APP may play a role in the regulation of vesicle fusion and synaptic function (Wang et al., 2005; Yang et al., 2007).

Rab3A was shown previously to be present in several different presynaptic vesicle types along with various other proteins, which are transported by different velocities along the axon including syntaxin-1, SNAP25, bassoon, piccolo, Munc18, N-Cadherin, and VAMP2 (Okada et al., 1995; Zhai et al., 2001), which are transported by different velocities along the axon (Shapira et al., 2003). Likely, APP transport vesicles represent only a subset of the cargo proteins that can be cotransported with Rab3A. Our data suggest that Rab3A may be involved in the packaging of specific proteins into different vesicle subtypes that in turn may be moved by specific motor proteins. In this regard, it is remarkable that inhibition of Rab3A had no influence on the anterograde transport of synaptophysin, although Rab3 colocalizes with synaptophysin at nerve terminals (Li et al., 1996a), and has been copurified from synaptic vesicles in immunoprecipitation experiments (Fischer von Mollard et al., 1990; de Wit et al., 1999). The anti-Rab3 antibody used in these two studies does not allow differentiating between the Rab3 isoforms. Thus, the apparent discrepancy between these studies and our analyses might be explained by the fact that synaptophysin is not a cargo of vesicles containing Rab3A but possibly a cargo of Rab3B–Rab3D-containing vesicles. Alternatively, Rab3A and synaptophysin may be packaged in a common vesicle after they have reached the nerve terminal in distinct transport vesicles, as suggested by previous immunoprecipitation studies (Okada et al., 1995). Consistent with this latter possibility, some components of the APP transport vesicle have been found in association with synaptic vesicle fractions (synapsin-1, syntaxin-1, SNAP25, etc.), although APP has not been found as a component of synaptic vesicles (Takamori et al., 2006).



**Figure 8.** Model of APP anterograde transport vesicle assembly. Data presented here suggest that, in neurons, APP is sorted into an FAT vesicle containing Rab3A and other presynaptic components. **1**, In the first step (likely in the late Golgi apparatus), the GTP-bound form of Rab3A (red circle) mediates packaging and assembly of APP and a specific set of presynaptic components (i.e., syntaxin-1, SNAP25, synapsin-1, Munc13-1, and RIM2) in a common vesicle. **2**, After this, Rab3A-GTP hydrolysis catalyzed by dimerized Rab3GAP p130 and p150 (yellow rectangle) causes a conformational change of Rab3A-GTP (red circle) to Rab3A-GDP (red square), accompanied by recruitment of a specific kinesin-1 isoform variant (kinesin-1C) to the APP transport vesicle. **3**, Finally, the assembled FAT vesicle is transported by this conventional kinesin via microtubule tracks to its target membrane at the presynaptic terminal, in which the APP transport vesicle fuses with the presynaptic acceptor membrane.

### Rab3A GTPase activity is required for APP transport

By using a Rab3A GTPase-deficient mutant and by silencing of Rab3GAP p130 or p150, we showed that conversion of Rab3A-GTP to Rab3A-GDP is essential for anterograde FAT transport of APP in neuronal cells. These findings are consistent with previous experiments in isolated axoplasm indicating that FAT depends on the activity of small GTPases (Bloom et al., 1993). Rab3 GTPase family members are key regulators of presynaptic vesicular transport with overlapping, but not identical, functions (Burstein et al., 1993; Deneka et al., 2003; Schluter et al., 2004; Ali and Seabra, 2005; Star et al., 2005). After delivery to their respective membranes, Rab3s are activated by replacement of GDP by GTP. GTP-bound Rab3s are thought to orchestrate the assembly of cargo contents, motor association, and docking interactions between donor and target membranes, whereas Rab3-GDP is presumably generated during or after exocytosis, allowing Rab3 release from the target membrane (Fischer von Mollard et al., 1991; Stahl et al., 1994; Sakisaka et al., 2002; Star et al., 2005). Recent data, however, showed that Rab3A knockdown, knock-out, or overexpression of GTP-locked mutant Rab3A significantly decreases the number of vesicles docked at the plasma membrane without altering the kinetics of individual exocytotic events (Schluter et al., 2006; Tsuboi and Fukuda, 2006; Coleman et al., 2007; Handley et al., 2007; van Weering et al., 2007). Together with these studies, our data argue that Rab3A does not play an essential role in vesicle fusion but rather in the transport of vesicles to the plasma membrane. In addition, our studies suggest that Rab3A-GTP hydrolysis might take place before the vesicle docks to the target membrane.

The well established role of Rab3s in protein sorting led us to examine alterations in the composition of APP-containing mem-

brane cargoes induced by reduced Rab3A activity. Significantly, we found reduced levels of kinesin-1C and other protein cargoes in APP-containing membranous organelles obtained from p130<sup>-/-</sup> mouse brain, in which Rab3A GTPase activity is inhibited. Together, our findings suggest a model in which Rab3 GTPase activity is required for correct assembly of APP and other membrane proteins (i.e., syntaxin-1, SNAP25, and synapsin-I) in a biochemically distinct transport vesicle type. In addition, our results suggest that Rab3A may regulate the association of kinesin-1C to the APP transport vesicle (Fig. 8).

### APP can be cleaved by $\alpha$ -secretase in transport vesicles

The subcellular compartment and spatial regulation of APP processing secretases, such as ADAM10, ADAM17, BACE1, or PS1, remains to be determined, because this information is critical for understanding the biology of APP. Early suggestions that BACE1 and PS1 are cotransported with APP in anterogradely transported axonal vesicles (Kamal et al., 2001) were not confirmed in subsequent studies (Lazarov et al., 2005; Goldsbury et al., 2006). Our studies here found that APP/kinesin-1-immunoprecipitated membrane fractions did not contain detectable levels of ADAM17, BACE1, PS1, or Nicastrin. Of the secretase components examined, only ADAM10 was detected in these membrane preparations. Remarkably, sAPP was detected in the isolated membrane fractions after *in vitro* incubations. Generation of this sAPP was blocked by a zinc chelator/inhibitor of  $\alpha$ -secretase, consistent with the possibility that  $\alpha$ -secretase activity is present in the immunoprecipitated organelle fractions. However, compared with full-length APP, the amount of sAPP was <1%, indicating that only low amounts of sAPP might be generated on its way to the plasma membrane. Alternatively, the process of immunoprecipitation may lead to activation of associated ADAM10. The physiological significance of this processing in immunoprecipitated vesicles remains to be determined, but therapeutic strategies affecting APP packaging in transport vesicles might help reduce A $\beta$  generation.

Together, our data suggest a novel, Rab3A-dependent mechanism for the coordinated regulation of APP sorting and axonal transport events, giving important insights into the physiological and pathogenic function of APP.

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