

# Palmitoylation controls trafficking of GAD65 from Golgi membranes to axon-specific endosomes and a Rab5a-dependent pathway to presynaptic clusters

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Accepted 1 December 2003

Journal of Cell Science 117, 2001-2013 Published by The Company of Biologists 2004

doi:10.1242/jcs.01030

## Summary

The GABA-synthesizing enzyme GAD65 is synthesized as a soluble cytosolic protein but undergoes post-translational modification(s) to become anchored to the cytosolic face of Golgi membranes before targeting to synaptic vesicle membranes in neuroendocrine cells. Palmitoylation of cysteines 30 and 45 in GAD65 is not required for targeting to Golgi membranes but is crucial for post-Golgi trafficking to presynaptic clusters in neurons. Here, we show that palmitoylated GAD65 colocalizes with the small GTP-binding protein Rab5a in Golgi membranes and in axons but not in dendrites. In the presence of the constitutively positive mutant Rab5(Q79L) palmitoylation resulted in polarized targeting of GAD65 to giant Rab5a-positive axonal endosomes, characterized by the absence of the Rab5a-effector molecule EEA1 and the transferrin receptor. By contrast, Rab5a-positive/EEA1-positive somatodendritic giant endosomes containing the transferrin receptor were devoid of GAD65. Palmitoylation-deficient GAD65 was excluded from

endosomal compartments. A dominant negative mutant of Rab5a, Rab5a(S34N), specifically blocked axonal trafficking and presynaptic clustering of palmitoylated GAD65, but did not affect axonal trafficking of mutants of GAD65 that fail to traffic to giant axonal endosomes containing Rab5a(Q79L). Two transmembrane synaptic vesicle proteins, VAMP2 and VGAT also localized to the axonal giant endosomes, and their axonal trafficking and presynaptic clustering was blocked by Rab5a(S34N). The results suggest that palmitoylation of GAD65 regulates the trafficking of the protein from Golgi membranes to an endosomal trafficking pathway in axons that is dependent on Rab5a and is required for the targeting of several synaptic vesicle proteins to presynaptic clusters.

Supplemental figures available online

Key words: Polarized sorting, Rab5a, Axonal trafficking, Presynaptic clusters, Palmitoylation

## Introduction

The sorting of newly synthesized membrane proteins to distinct domains in polarized cells appears to begin in the Golgi/trans Golgi network (TGN), where proteins can segregate and exit in separate transport vesicles (Keller et al., 2001). While targeting of proteins to apical vs basolateral surfaces in epithelial cells sometimes occurs directly from the TGN, the trafficking pathways are often indirect. They can involve additional sorting steps at the plasma membrane and/or in early endosomes and transcytosis from one domain to another before a final destination is reached (reviewed in Keller and Simons, 1997). Originally it was suggested that basolateral and apical domains of epithelial cells correspond to the somatodendritic and axonal surfaces of neurons, respectively (Dotti and Simons, 1990). However, this only holds true to an approximation. While sorting of basolateral and dendritic proteins may use some of the same elements, most apical proteins do not target selectively to axons but are found in both axons and dendrites. Thus separate targeting signals, mechanisms and pathways might have evolved to achieve the complex functional integrity of neuronal synapses. In neurons, some proteins seem to follow a direct pathway from the TGN to the somatodendritic surface (reviewed in Winckler

and Mellman, 1999) but sorting to axons, followed by transcytosis to the somatodendritic domain, was observed for the Alzheimer's disease precursor protein (Tienari et al., 1996). The pathways involved in targeting of proteins to presynaptic termini in axons remain obscure. For instance, it is unclear whether axonal proteins are sorted in axonally directed transport vesicles directly at the TGN or whether they undergo additional sorting steps. Targeting of membrane proteins to the axonal surface, can occur by at least two distinct mechanisms. For instance, the protein NgCAM contains trafficking signal(s), which mediate its sorting into carriers that preferentially traffic to the axonal membrane (selective delivery). By contrast, the protein VAMP2 is delivered to both the surface of axons and dendrites but is preferentially endocytosed from the latter membrane and its preferential axonal localization is dependent on endocytosis (selective retention) (Sampo et al., 2003). At least two distinct types of transport vesicles carry membrane proteins to axons (Kaether et al., 2000) and axonal vesicles containing distinct motor proteins contain different complements of synaptic vesicle proteins (Okada et al., 1995). Thus neurons appear to utilize a variety of membrane transport pathways to achieve the precise localization of newly synthesized proteins in axons.

GAD65, the smaller isoform of the GABA-synthesizing enzyme glutamate decarboxylase, targets to presynaptic clusters in axons and exhibits relative dendritic exclusion (Kanaani et al., 1999; Kanaani et al., 2002). GAD65 is synthesized as a soluble hydrophilic molecule but undergoes N-terminal post-translational modification(s) resulting in targeting to the cytosolic leaflet of Golgi membranes and a subsequent trafficking to presynaptic clusters (Kanaani et al., 2002 and references therein). The palmitoylated N-terminal domain in GAD65, amino acid (aa) residues 1-60, selectively targets soluble proteins and their associated partners to presynaptic clusters. Three separate targeting signals are required for this process. They include a Golgi targeting signal residing within aa residues 1-23, a signal for membrane anchoring residing within aa residues 24-31 and a palmitoylation motif involving cysteines 30 and 45. The first two signals are required for targeting to Golgi membranes. Palmitoylation is not required for anchoring to Golgi membranes but is crucial for the efficient targeting of GAD65 from Golgi membranes to presynaptic clusters (Kanaani et al., 2002).

Early endosomes are important sorting compartments for newly synthesized and recycling membrane proteins (reviewed in Keller and Simons, 1997). Rab5a is a limiting regulator of early steps of endocytosis (Bucci et al., 1992). Rab5a regulates heterotypic fusion of nascent endocytic vesicles with early endosomes and homotypic fusion of early endosomes (reviewed in Novick and Zerial, 1997). Furthermore, Rab5a has a role in regulating motility of early endosomes on microtubules (Nielsen et al., 1999). Rab5a regulates the binding of several effector molecules to early endosomes including the early endosomal autoantigen EEA1 (Christoforidis et al., 1999). In hippocampal neurons, Rab5a is associated with both somatodendritic and axonal endosomal vesicles and is detected in the membrane of synaptic vesicles (de Hoop et al., 1994). EEA1 is only associated with Rab5a in somatodendritic vesicles, while Rab5a containing vesicles in axons are devoid of EEA1 and appear to contain a distinct effector complex (Wilson et al., 2000).

In this study, we show that palmitoylation controls the entry of GAD65 into a trafficking pathway that involves Rab5a and results in polarized targeting to axon specific endosomes.

## Materials and Methods

### Antibodies

The following antibodies were used in the immunofluorescence experiments: mouse monoclonal antibodies against EEA1, Golgi matrix protein 130 (GM130) (both BD Biosciences), microtubule associated protein 2 (MAP2) (Sigma), HA epitope tag (BABCO) and GAD65 [GAD6 (Chang and Gottlieb, 1988)], and rabbit polyclonal antibodies against synaptophysin (Zymed) and vesicular GABA transporter [(VGAT) (McIntire et al., 1997) a gift from Robert Edwards, UCSF]. A chicken polyclonal antibody against purified 6-His-tagged green fluorescent protein (GFP) was from Chemicon. Cy3-conjugated donkey antibodies against mouse and rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 350 goat anti-mouse antibodies and Alexa Fluor 488 goat antibodies against rabbit, mouse, and chicken IgG were from Molecular Probes (Eugene, OR).

### DNA constructs

Chimeras containing enhanced green fluorescent protein (EGFP) at the C-terminal end of wt GAD65, a palmitoylation-deficient mutant,

GAD65(C30,45A), a Golgi-targeting mutant, 24-60GAD65, and wt PSD-95 were described earlier (Kanaani et al., 2002). Wt and mutant forms of Rab5a (a gift from Philip D. Stahl, Washington University, St Louis, MO) were HA-tagged at the N-terminus and subcloned in the plasmid GW1. The chimeras containing EGFP at the N-terminus of wt and dominant negative mutant (S34N) of human Rab5a in pEGFP-C3, were provided by Ruth Collins, Cornell University, NY. The C-terminal fusion protein of EGFP and the human transferrin receptor (TfR) in the mammalian expression vector JPA5 was provided by Gary Banker, Oregon Health Sciences University, Portland, OR. The C-terminal fusion protein of the HA-tag and VGAT in the mammalian expression vector pcDNA3 was from Robert Edwards. The fusion protein containing the pH sensitive mutant of GFP (ecliptic pHluorin), joined to VAMP2/synaptobrevin at its lumenally exposed C-terminus (synapto-pHluorin) in the pCI mammalian expression vector, was kindly provided by James Rothman, Memorial Sloan Kettering Cancer Center, NY (Miesenböck et al., 1998).

### Cell culture

Preparation, culture and transfection of rat primary hippocampal neurons were carried out as described (Kanaani et al., 2002). Neurons were fixed 24 or 72 hours after transfection. Culture and transfection of COS-7 cells were carried out as described previously (Shi et al., 1994; Namchuk et al., 1997).

### Immunofluorescence analyses and quantitation

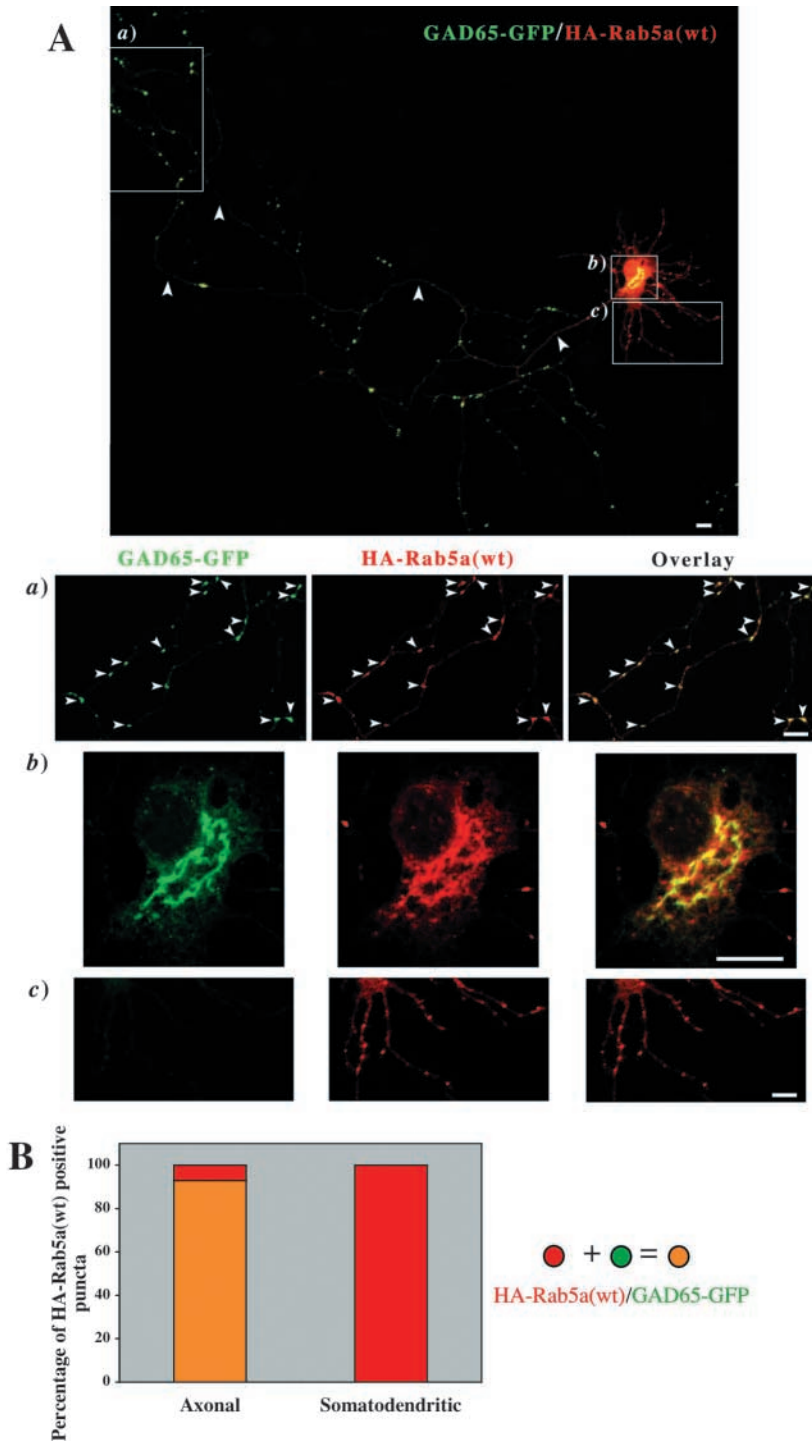
For indirect immunofluorescence, neuronal cultures were fixed in either 2% paraformaldehyde in PBS (pH 7.4) or methanol ( $-20^{\circ}\text{C}$ ) (staining for synaptophysin). COS-7 cells were fixed 18-24 hours after transfection with 2% paraformaldehyde. A chicken anti-GFP antibody (Chemicon) was used as a primary antibody to enhance the signal of GFP-chimeras in neuronal transfections. Primary antibodies were added in blocking solution for 1 hour at room temperature followed by incubation with appropriate fluorochrome-conjugated secondary antibodies diluted in blocking solution for 1 hour at room temperature. After three washes with Tris-buffered saline (TBS), the coverslips were mounted on slides with Fluoromount-G (Southern Biotechnology Associates). Fluorescent images were obtained using a Leica TCS NT laser scanning confocal microscope with a Krypton-Argon laser (Leica). All confocal images derived from eight consecutive horizontal optical sections of an estimated thickness of 0.2-0.5  $\mu\text{m}$ .

Projections of the eight layers were used for quantitation of the number of axonal puncta for wt and palmitoylation-deficient GAD65-GFP in the presence of wt Rab5a or Rab5a(S34N). Metamorph imaging software (Universal Imaging Corp.) was used to determine the number of puncta detected after thresholding the image. The threshold was set to be twice the axonal background. For multi-channel imaging of axonal and dendritic puncta to quantitate colocalization of wt or palmitoylation-deficient GAD65 with wt Rab5a and Rab5a(Q79L), respectively, fluorescent dyes were imaged sequentially in frame-interlace mode, to eliminate cross talk between the channels.

## Results

### Wt GAD65 colocalizes with Rab5a in axons but not in dendrites and palmitoylation is required for this colocalization

Presynaptic clustering is decreased to <5% of wt in palmitoylation-deficient GAD65 (Kanaani et al., 2002). We sought to identify steps in the post-Golgi trafficking pathway of GAD65 to presynaptic clusters and address the question where wt and palmitoylation-deficient GAD65 diverge in the pathway.



Immunoelectron microscopy has revealed GAD65 in the membrane of synaptic-like microvesicles in pancreatic  $\beta$  cells (Christgau et al., 1992) and its targeting to presynaptic clusters is consistent with a localization in synaptic vesicle membranes in neurons. Because synaptic vesicles appear to form de novo from early endosomes (reviewed in Hannah et al., 1999), we first addressed the question whether GAD65 colocalizes with the early endosomal marker Rab5a in primary neurons. Coexpression of HA-Rab5a(wt) and wt GAD65-GFP showed their colocalization in presynaptic clusters in axons (Fig. 1A, enlarged

**Fig. 1.** Palmitoylated wt GAD65-GFP colocalizes with wt HA-Rab5a in Golgi membranes and in axon termini but not in somatodendritic puncta.

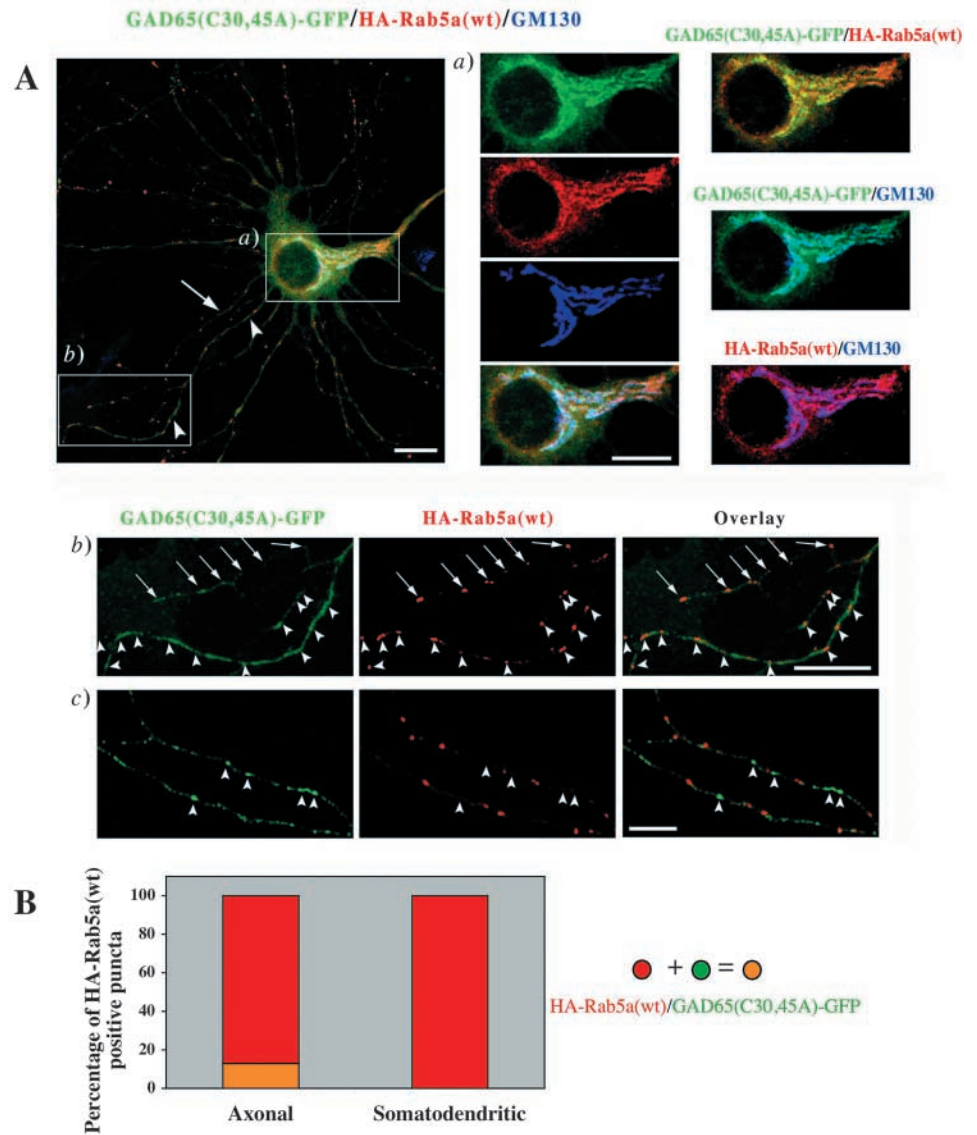
(A) Confocal images of a hippocampal neuron co-transfected with plasmids encoding wt GAD65-GFP and HA-tagged wt Rab5a. Neurons were double immunolabeled for GFP and HA. GAD65-GFP colocalizes with wt Rab5a in axonal puncta (arrowheads, enlarged frame *a*) and in Golgi membranes (enlarged frame *b*) but not in dendritic puncta (enlarged frame *c*). Scale bars, 10  $\mu$ m. (B) The percentage of HA-Rab5a(wt)-positive puncta expressing wt GAD65-GFP was analyzed as described in Materials and Methods. The yellow color indicates the percentage of HA-Rab5a(wt)-positive puncta expressing wt GAD65-GFP.

frame *a* and Fig. 1B) and in the Golgi compartment in the soma (Fig. 1A, enlarged frame *b*). Therefore, >90% of GAD65-GFP-positive axonal puncta contained HA-Rab5a(wt) and vice versa. GAD65 was, however, completely absent from Rab5a-positive somatodendritic endosomes (Fig. 1A, enlarged frame *c* and Fig. 1B). We have previously shown that the distribution of wt GAD65-GFP and endogenous GAD65 is similar in primary neurons (Kanaani et al., 2002). We addressed the question whether endogenous GAD65 colocalizes with GFP-Rab5a(wt). Primary neuronal cultures were transfected with GFP-Rab5a(wt) and double immunostained for GFP and for endogenous GAD65 using the monoclonal antibody GAD6. Endogenous GAD65 in transfected GABA-ergic neurons colocalized with GFP-Rab5a(wt) in the Golgi compartment and in presynaptic clusters in axons (see supplemental data Fig. S1, enlarged frames *a,b*, <http://jcs.biologists.org/supplemental/>). By contrast, none of the GFP-Rab5a(wt)-positive somatodendritic puncta contained endogenous GAD65 (see supplemental data Fig. S1, enlarged frames *a,b*). Thus both endogenous GAD65 and GAD65-GFP colocalize with Rab5a(wt) in Golgi membranes and in presynaptic clusters but are completely absent from somatodendritic puncta containing Rab5a(wt).

The palmitoylation-deficient mutant GAD65(C30,45A)-GFP colocalized with HA-Rab5a (wt) in Golgi membranes (Fig. 2A, enlarged frame *a*). Palmitoylation-deficient GAD65 has a diffuse appearance in dendrites and proximal axons (Fig. 2A, enlarged frame *b*) but

is detected in rare puncta in the most distal part of axons (Fig. 2A, enlarged frame *c*). GAD65(C30,45A)-GFP was completely absent from Rab5a-positive somatodendritic endosomes (Fig. 2B). Furthermore, almost all of the rare axonal puncta that expressed GAD65(C30,45A)-GFP were devoid of HA-Rab5a(wt) (Fig. 2, enlarged frame *c*). GAD65(C30,45A)-GFP was detected in <15% of HA-Rab5a(wt)-positive axonal puncta (Fig. 2B). Therefore, palmitoylation is crucial for the efficient targeting of GAD65 to Rab5a-positive presynaptic clusters.

**Fig. 2.** Palmitoylation-deficient GAD65 colocalizes with Rab5a in Golgi membranes but not in axonal or dendritic puncta. (A) Confocal images of a hippocampal neuron co-transfected with plasmids encoding palmitoylation-deficient GAD65-GFP and HA-tagged wt Rab5a. Neurons were triple immunolabeled for GFP, HA, and the Golgi marker GM130. Arrowheads, axon; arrows, dendrites; scale bars, 10  $\mu$ m. Palmitoylation-deficient GAD65(C30,45A)-GFP colocalizes with wt Rab5a in Golgi membranes immunostained for GM130 (enlarged frame *a*, bottom frame in the middle shows overlay of the three colors, frames to the right show overlay of two colors). GAD65(C30,45A) is mostly diffuse in axons and dendrites and does not colocalize with wt Rab5a in axonal (arrowheads) or dendritic (arrows) puncta (enlarged frame *b*). Frame *c* shows a view of a distal axon and reveals that the rare axonal puncta containing palmitoylation-deficient GAD65 are devoid of HA-Rab5a. (B) The percentage of HA-Rab5a(wt)-positive puncta expressing palmitoylation-deficient GAD65-GFP was analyzed as described in Materials and Methods. The yellow color indicates the percentage of HA-Rab5a(wt)-positive puncta expressing GAD65(C30,45A)-GFP.



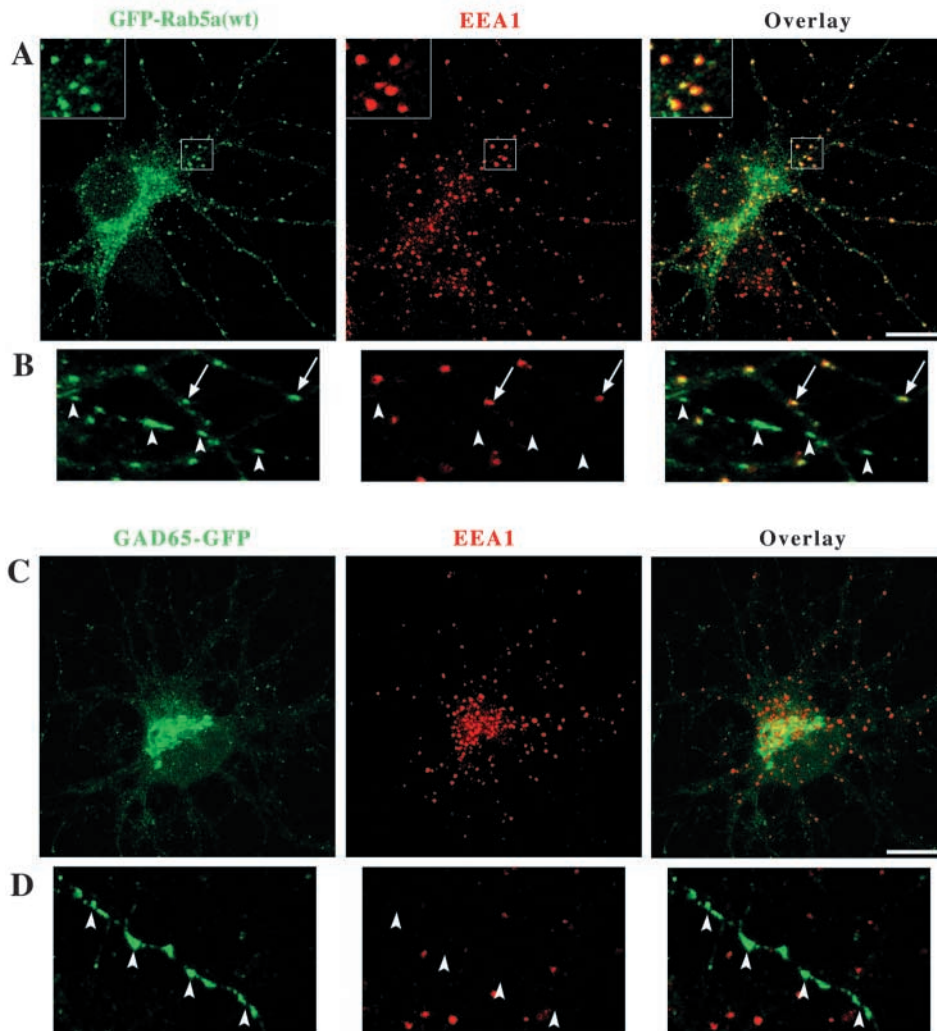
### GAD65 is excluded from endosomes containing the Rab5a effector EEA1

EEA1 selectively targets to Rab5a-positive somatodendritic endosomes but is absent from axonal puncta and presynaptic clusters containing Rab5a, which suggests a polarized distribution of Rab5a effector complexes (Fig. 3A,B) (Wilson et al., 2000). Transfection of GAD65-GFP into hippocampal neurons revealed a complete lack of colocalization with endogenous EEA1 (Fig. 3C,D). Similarly, palmitoylation-deficient GAD65 did not colocalize with endogenous EEA1 (results not shown). Thus, GAD65 is excluded from somatodendritic endosomal vesicles containing Rab5a associated with an effector complex containing EEA1.

### Palmitoylation results in polarized targeting of wt GAD65 to Rab5a-positive/EEA1 negative axonal vesicles of endosomal origin

Immunostaining for synaptophysin, confirmed that the axonal clusters where wt GAD65 and wt axonal Rab5a colocalize are

presynaptic termini (not shown). We addressed the question whether palmitoylated GAD65 is targeted to early endosomes. The Rab5a(Q79L) mutant is blocked in the GTP-bound state and its expression results in the formation of giant early-endosomal vesicles (Roberts et al., 1999). Expression of Rab5a(Q79L) in primary neurons resulted in a decrease in the number of presynaptic clusters and in its appearance in giant axonal endosomes (which lack EEA1 and line the entire axon) and in giant somatodendritic endosomes containing EEA1 (Figs 4, 5). While wt Rab5a localized to the Golgi compartment (Figs 1, 2), the Rab5a(Q79L) mutant was distinctly absent from Golgi membranes (Fig. 4A,B). Instead, in the soma, this mutant was exclusively detected in giant endosomes (Fig. 4A,B). This was in stark contrast to wt and palmitoylation-deficient GAD65 in the soma. They remained predominantly located in the Golgi compartment in the presence of the HA-Rab5a(Q79L) mutant and were completely absent from somatodendritic endosomes, containing HA-Rab5a(Q79L) (Fig. 4A,B). However, wt GAD65-GFP colocalized with HA-Rab5a(Q79L) in ~90% of the giant axonal endosomes that were negative for EEA1 (Fig. 5A-C,E).



**Fig. 3.** EEA1-positive somatodendritic early endosomes are devoid of GAD65. Confocal images of hippocampal neurons expressing either GFP-tagged wt Rab5a or GAD65-GFP and immunolabeled for GFP and endogenous EEA1. (A) Wt Rab5a colocalizes with EEA1 in early endosomes in the soma and proximal dendrites (enlarged frame). (B) Enlarged view of a distal axon (arrowheads) and distal dendrites (arrows) of the neuron shown in A, reveals colocalization of Rab5a and EEA1 in dendritic puncta and lack of EEA1 in axonal puncta containing Rab5a. (C) GAD65 is absent from somatodendritic early endosomes containing EEA1. (D) EEA1 is absent from presynaptic clusters (arrowheads) containing GAD65-GFP. Scale bars, 10  $\mu$ m.

2002) and is excluded from Rab5a(Q79L)-containing vesicles in soma, dendrites, and axons (Fig. 6). Therefore, targeting to Golgi membranes appears to be a prerequisite for the sorting of GAD65 to axonal endosomes.

We next addressed the question whether polarized targeting to axonal endosomes is a general property of synaptic vesicle membrane proteins. The vesicular GABA transporter VGAT and another transmembrane synaptic vesicle protein, VAMP2, showed complete colocalization with GAD65 and wt Rab5a in presynaptic clusters (see supplemental data Fig.

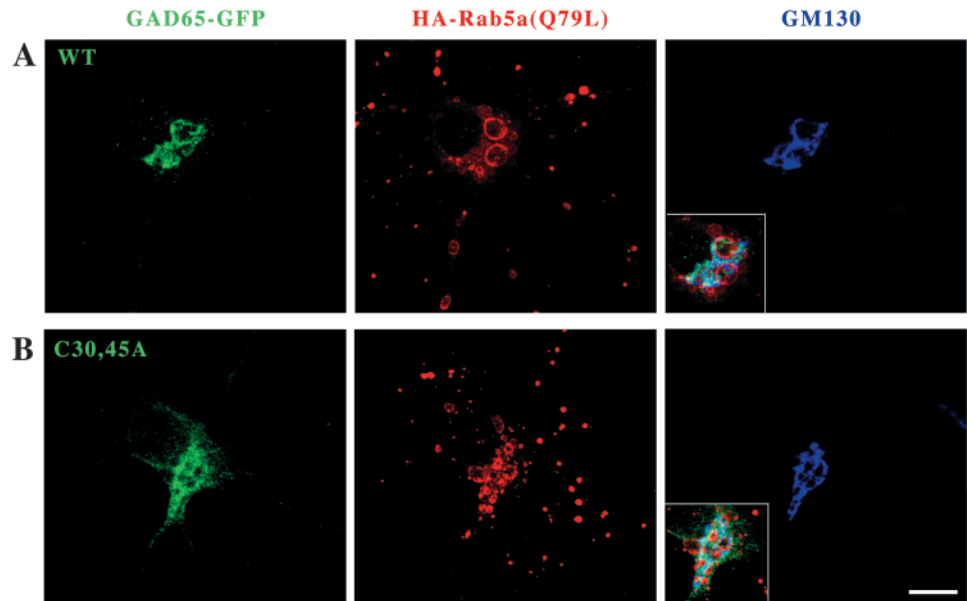
S3, <http://jcs.biologists.org/supplemental/> and results not shown). While VGAT shared the relative absence from dendrites with GAD65, VAMP2 colocalized with wt Rab5a in dendritic puncta (results not shown). VGAT and VAMP2 were coexpressed with Rab5a(Q79L) in primary hippocampal neurons. Those experiments revealed that VGAT and VAMP2 colocalize with Rab5a(Q79L) in both somatodendritic and axonal giant endosomes (see supplemental data Fig. S4, <http://jcs.biologists.org/supplemental/>). Thus, both VGAT and VAMP2 are targeted to axonal endosomes as is palmitoylated GAD65, while the exclusive targeting of the latter to axonal endosomes is a distinct feature. VAMP2 was shown to be delivered to the surface of both axons and dendrites, but preferably endocytosed from the dendritic membrane (Sampo et al., 2003). VGAT is also a transmembrane protein and as such might traffic to the plasma membrane before being sorted to synaptic vesicles. It is therefore possible that VGAT and VAMP2 in somatodendritic endosomes represent proteins that were endocytosed from dendritic plasma membranes. Along those lines, the absence of GAD65 in somatodendritic endosomes is consistent with it not being delivered to the dendritic surface but rather being selectively targeted to axonal endosomes.

Similarly, in primary neuronal cultures only transfected with GFP-Rab5a(Q79L), endogenous GAD65 in transfected GABAergic neurons localized to axonal endosomes containing GFP-Rab5a(Q79L) (see supplemental data Fig. S2, enlarged frames *a,b*, <http://jcs.biologists.org/supplemental/>) but was completely absent from somatodendritic endosomes containing GFP-Rab5a(Q79L) (see supplemental data Fig. S2, enlarged frames *a,c*).

Palmitoylation-deficient GAD65 was also excluded from HA-Rab5a(Q79L)-positive somatodendritic giant endosomes (results not shown). By contrast to wt GAD65-GFP and endogenous GAD65, palmitoylation-deficient GAD65 was largely absent from Rab5a(Q79L)-positive axonal endosomes (Fig. 5D,E). In sum, palmitoylation is required for the efficient trafficking of GAD65 to axonal endosomes and the protein is excluded from somatodendritic early endosomes.

The palmitoylated 1-60 aa region of GAD65 mediates Golgi targeting and presynaptic clustering of soluble proteins. However, removal of a Golgi-localization signal in the first 24 aa residues results in protein 24-60GAD65-GFP that is palmitoylated but targets to membranes distinct from the Golgi compartment. This protein is in both axonal and dendritic puncta but fails to reach presynaptic clusters (Kanaani et al.,

**Fig. 4.** Lack of colocalization of the GTP-bound Rab5a(Q79L) mutant with either wt or palmitoylation-deficient GAD65 in the soma of primary neurons. Confocal images of the soma of hippocampal neurons co-transfected with plasmids encoding either wt (A) or palmitoylation-deficient GAD65-GFP (B) together with HA-tagged constitutively active mutant of Rab5a(Q79L). Neurons were triple immunolabeled for GFP, HA, and the Golgi marker GM130. (A,B) Neither Wt GAD65-GFP nor palmitoylation-deficient GAD65-GFP are detected in the giant endosomes in the soma containing Rab5a(Q79L). Both Wt GAD65-GFP and palmitoylation-deficient GAD65-GFP are localized in the Golgi compartment, immunostained for GM130 but devoid of Rab5a(Q79L). Scale bar, 10  $\mu$ m.



#### Transient accumulation of wt, but not palmitoylation-deficient GAD65, together with dominant negative Rab5a(S34N) in neurite tips

The Rab5a(S34N) protein is a dominant negative mutant, which remains in the GDP-bound state and inhibits early endosome fusion and formation of endosome-derived vesicles. Stenmark et al. described the accumulation of Rab5a(S34N) in fusion-incompetent early endosomes in non-neuronal cells (Stenmark et al., 1994). Twenty four hours after co-transfecting primary hippocampal neurons, Rab5a(S34N) was detected in Golgi membranes together with wt GAD65 (see supplemental data Fig. S5, <http://jcs.biologists.org/supplemental/> and results not shown). In addition, both Rab5a(S34N) and wt GAD65 accumulated at the tips of dendrites (Fig. 7A) and axons (Fig. 7B). If both wt and palmitoylation-deficient GAD65 traffic to early endosomes and diverge later in the trafficking pathway, they would each be expected to accumulate in endocytic profiles in the presence of Rab5a(S34N). In 24-hour coexpression experiments, palmitoylation-deficient GAD65 was detected in Golgi membranes together with Rab5a(S34N) but was absent from accumulates of Rab5a(S34N) at the tips of dendrites (Fig. 7C) and axons (Fig. 7D). Similarly, while experiments in COS-7 cells revealed the colocalization of wt GAD65 with Rab5a(S34N) in Golgi membranes and in peripheral patches (see supplemental data Fig. S6ii-iii, <http://jcs.biologists.org/supplemental/>), the palmitoylation-deficient GAD65 only colocalized with Rab5a(S34N) in the Golgi compartment but was absent from the peripheral patches containing Rab5a(S34N) (see supplemental data Fig. S6iv-v). Surprisingly, endogenous EEA1, which associates with wt and Rab5a(Q79L) in early somatodendritic endosomes, was not detected in cells expressing Rab5a(S34N) (see supplemental data Fig. S7i, <http://jcs.biologists.org/supplemental/>), which suggests that the expression of EEA1 remains below detection level in the absence of Rab5a-GTP. Collectively, the results of coexpression experiments with Rab5a(Q79L) and Rab5a(S34N) presented above, suggest that the palmitoylation-deficient protein does not traffic to early endosomes. Thus wt

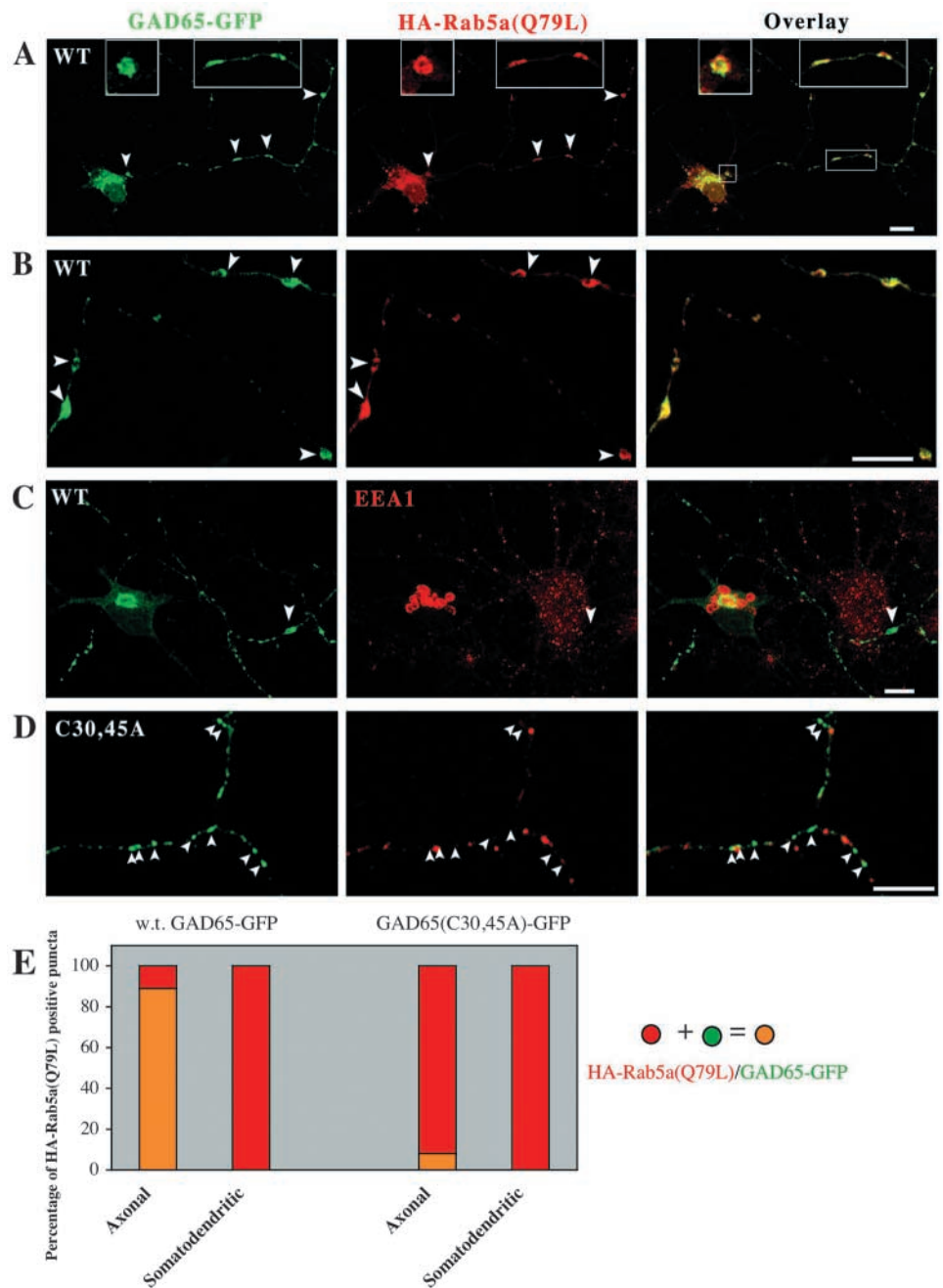
and palmitoylation-deficient GAD65 proteins diverge before trafficking of the former to early endosomes. We propose, that palmitoylation controls anterograde trafficking of GAD65 from Golgi membranes to early endosomes.

In the 24-hour transfection experiments, the detection of wt GAD65 together with Rab5a(S34N) at the tips of dendrites and axons, was in stark contrast to the relative absence of wt GAD65 in dendrites expressing wt Rab5a (see supplemental data Fig. S7ii-iii). The accumulation of wt GAD65 and Rab5a(S34N) in neurite tips was transient, because 72 hours after transfection the proteins were no longer detected in this location (Fig. 8A).

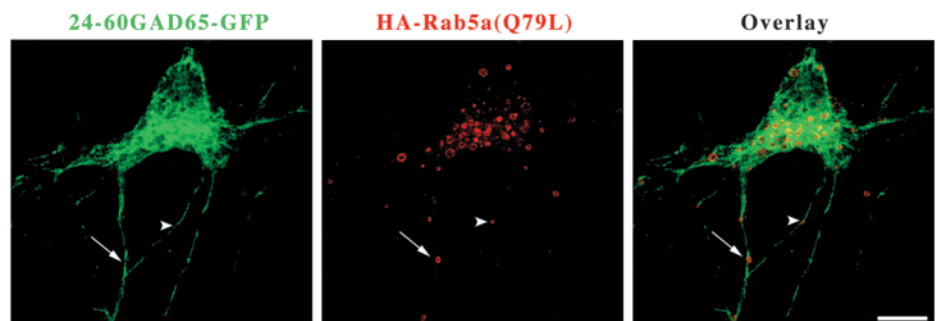
#### The Rab5a(S34N) mutant blocks axonal targeting and presynaptic clustering of wt GAD65-GFP and endogenous GAD65 but does not affect trafficking of the GAD65(C30,45A)-GFP and GAD65(24-60)-GFP proteins

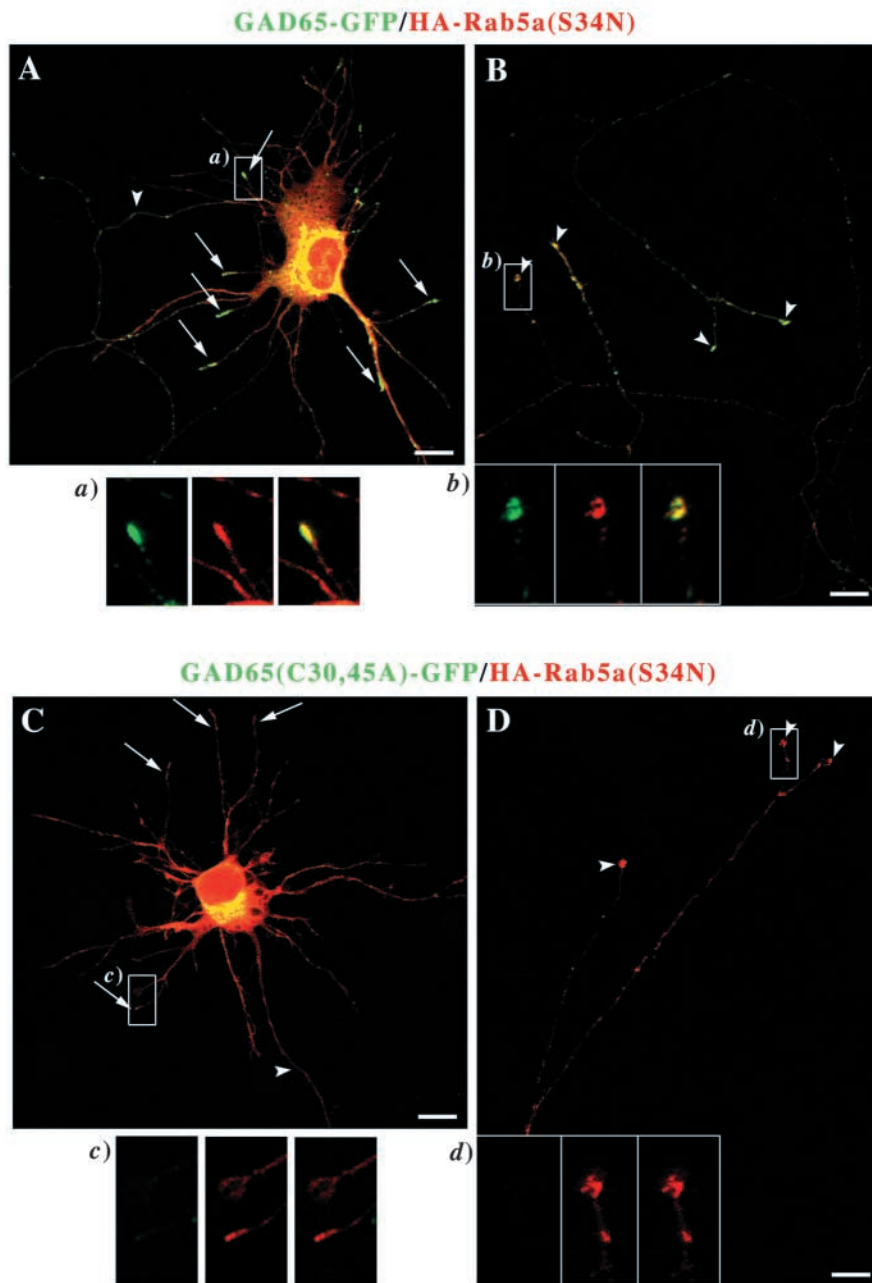
The expression of GAD65 with Rab5a(S34N) for 24 hours in neurons, drastically reduced presynaptic clustering of GAD65-GFP and both proteins accumulated at the tip of the axon. Seventy-two hours after transfecting neurons with GAD65-GFP and HA-Rab5a(S34N), analysis revealed that axonal targeting and presynaptic clustering of GAD65 were largely abolished (Fig. 8A); the average number of axonal puncta containing wt GAD65-GFP per neuron was  $272 \pm 18$  ( $n=7$ ) in the presence of HA-Rab5a(wt) and  $11 \pm 5$  ( $n=6$ ) in the presence of HA-Rab5a(S34N) in neuronal cultures of similar density. Similarly, in primary neuronal cultures transfected only with GFP-Rab5a(S34N) for 72 hours, axonal trafficking of endogenous GAD65 in transfected GABA-ergic neurons was largely abolished (see supplemental data Fig. S8, <http://jcs.biologists.org/supplemental/>). In 72 hour expression experiments GAD65-GFP, endogenous GAD65, HA-Rab5a(S34N), and GFP-Rab5a(S34N) were all primarily detected in the Golgi compartment of hippocampal neurons and accumulation in neurite tips was not observed (Fig. 8A,

**Fig. 5.** Palmitoylation results in targeting of GAD65 to Rab5a(Q79L)-positive/EEA1-negative axonal endosomes. Confocal images of hippocampal neurons co-transfected with plasmids encoding either wt (A-C) or palmitoylation-deficient GAD65-GFP (D) together with HA-tagged constitutively active mutant of Rab5a(Q79L). Neurons were double immunolabeled for GFP, and either HA or endogenous EEA1. (A) wt GAD65-GFP colocalizes with Rab5a(Q79L) in giant axonal vesicles (arrowheads) lining up the axon starting at the base. (B) Enlarged view of more distal axonal vesicles of the neuron shown in A shows the presence of wt GAD65 in axonal endosomes containing HA-Rab5a(Q79L). (C) EEA1, is present in giant endosomal vesicles in the cell body but absent from giant axonal vesicles containing wt GAD65. In the presence of Rab5a(Q79L), EEA1 remains confined to vesicles in the cell body, in contrast to the situation in a non-transfected cell (to the right) where it is also in dendrites. (D) A view of the most distal part of an axon expressing palmitoylation-deficient GAD65 (arrowheads). Axonal puncta containing the palmitoylation-deficient protein do not colocalize with axonal endosomes containing HA-Rab5a(Q79L). (E) The percentage of HA-Rab5a(Q79L)-positive puncta expressing wt GAD65-GFP or palmitoylation-deficient GAD65-GFP was quantified as described in Materials and Methods. The yellow color indicates the percentage of HA-Rab5a(Q79L)-positive puncta expressing wt GAD65-GFP or GAD65(C30,45A)-GFP, respectively. Scale bars, 10  $\mu$ m.



**Fig. 6.** The Golgi localization mutant, 24-60GAD65-GFP, is excluded from Rab5a(Q79L)-positive/EEA1-negative axonal endosomes. Confocal images of hippocampal neurons co-transfected with plasmids encoding HA-Rab5a(Q79L) and the 24-60GAD65-GFP mutant, which targets to vesicles in both dendrites and axons but fails to reach presynaptic clusters. Neurons were double immunolabeled for GFP and HA. The 24-60GAD65-GFP mutant, which is targeted to membranes distinct from Golgi, is absent from giant vesicles containing Rab5a(Q79L) in soma, dendrites (arrow) and axons (arrowhead). Scale bar, 10  $\mu$ m.





**Fig. 7.** Accumulation of wt but not palmitoylation-deficient GAD65 in neurite tips in the presence of the Rab5(S34N) mutant. Confocal images of hippocampal neurons 24 hours after transfection with plasmids encoding HA-tagged Rab5a(S34N) (A-D) and wt GAD65-GFP (A,B) or palmitoylation-deficient GAD65-GFP (C,D) and double immunolabeled for GFP and HA. Enlarged frames show (from left to right) immunolabeling for GFP, immunolabeling for HA, and overlay, respectively. (A) Wt GAD65 accumulates with Rab5a(S34N) in the tip of dendrites. (B) View of the distal axon of the neuron shown in A reveals accumulation of wt GAD65 together with Rab5a(S34N) at the tips of the branched axon. (C) Palmitoylation-deficient GAD65 does not accumulate with Rab5a(S34N) at the tip of dendrites. (D) View of the distal axon of the neuron shown in C reveals the absence of palmitoylation-deficient GAD65 in accumulates of Rab5a(S34N) at the tip of axons. Scale bars, 10  $\mu$ m.

mutant (Fig. 8C,D), suggesting that the expression of Rab5a(S34N) does not result in a general block of axonal or dendritic trafficking. Therefore, the specific inhibition of axonal transport and presynaptic clustering of palmitoylated GAD65, imposed by the dominant negative mutant Rab5a(S34N), strongly suggest a specific role of axonal early endosomes and axon-specific Rab5a in trafficking of newly synthesized palmitoylated wt GAD65 to presynaptic clusters.

#### Rab5a(S34N) blocks presynaptic clustering of several synaptic vesicle proteins

Neurite outgrowth and the general appearance of neurons was not affected by the expression of Rab5a(S34N) for 24 or 72 hours. This is consistent with the normal synaptic ultrastructure reported for *Drosophila melanogaster* expressing Rab5a(S34N) (Wucherpfennig et al., 2003). However, immunostaining for endogenous synaptophysin in the 72-hour experiments with Rab5a(S34N) did not show the location in typical presynaptic clusters (results not shown). We analyzed the targeting of the transmembrane synaptic vesicle proteins VGAT-HA and VAMP2-GFP in 72-hour coexpression experiments with Rab5a(S34N). In the presence of Rab5a(S34N), VGAT-HA remained confined to Golgi membranes where it colocalized with the Rab5a mutant (Fig. 9A); axonal trafficking and presynaptic clustering were largely abolished (Fig. 9B). Similarly, axonal targeting and presynaptic clustering of VAMP2-GFP was largely blocked in the presence of Rab5a(S34N) (results not shown).

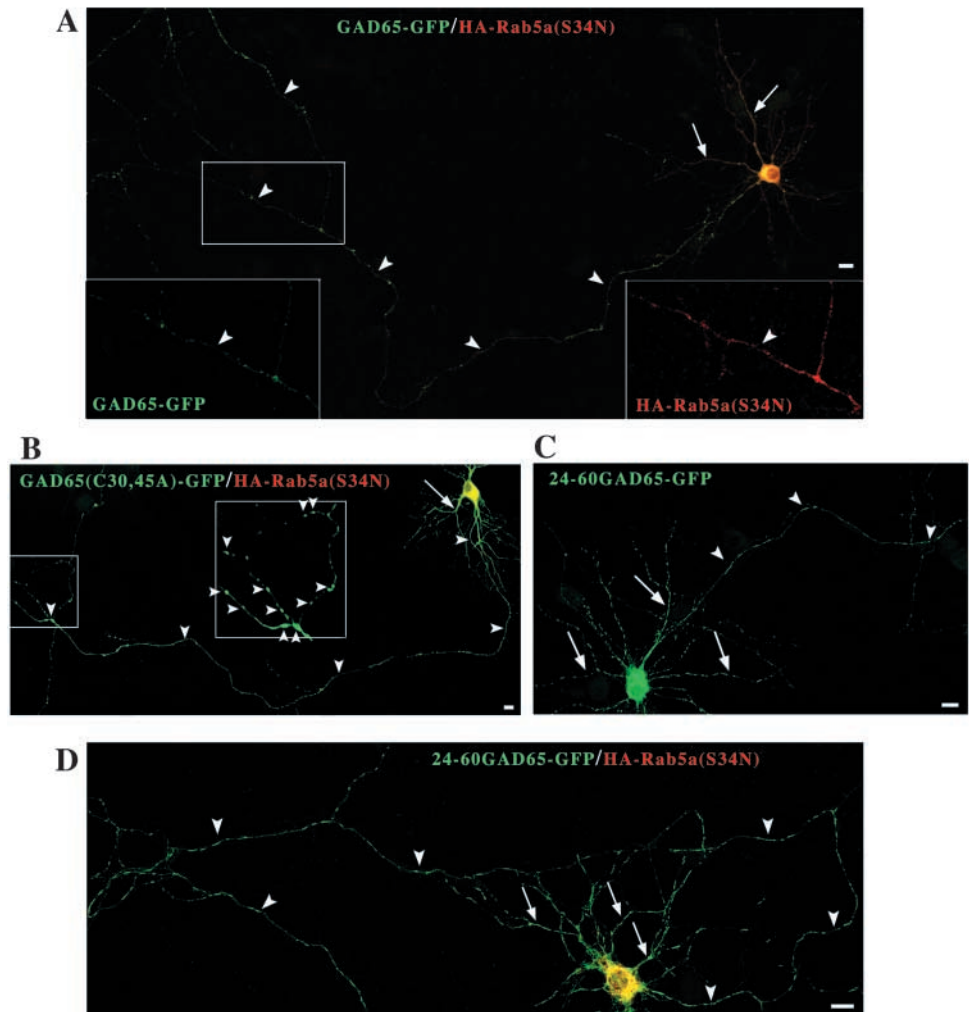
Therefore, targeting to presynaptic clusters of at least three synaptic vesicle proteins, GAD65, VGAT and VAMP2, appears

see supplemental data Fig. S5 and S8, and results not shown). Therefore, Rab5a(S34N) appears to first compromise the preferential targeting of GAD65 to axons and then induce a block in targeting from Golgi membranes to the periphery.

The dominant negative Rab5a(S34N) mutant did not affect the subcellular localization of the palmitoylation-deficient mutant of GAD65. The average number of axonal puncta containing GAD65(C30,45A)-GFP per neuron was  $25 \pm 6$  ( $n=6$ ) in the presence of wt Rab5a and  $17 \pm 7$  ( $n=6$ ) in the presence of Rab5a(S34N) in neuronal cultures of similar density. Thus in the presence of Rab5a(S34N), palmitoylation deficient GAD65 remained predominantly located in the Golgi compartment and diffuse in dendrites and a small fraction was still detected in axonal puncta (Fig. 8B). The Rab5a(S34N) mutant did not affect axonal or dendritic targeting of the 24-60GAD65-GFP



**Fig. 8.** The Rab5a(S34N) dominant negative mutant inhibits axonal trafficking of wt GAD65-GFP but does not affect trafficking of palmitoylation-deficient GAD65 or of the 24-60GAD65-GFP protein to axons and dendrites. Confocal images of hippocampal neurons 72 hours after transfection with plasmids encoding HA-tagged Rab5a(S34N) and either wt GAD65-GFP, palmitoylation-deficient GAD65-GFP, or a targeting mutant, 24-60GAD65-GFP, which targets to vesicles in both dendrites and axons but fails to reach presynaptic clusters. (A) Axonal trafficking and presynaptic clustering of wt GAD65-GFP is almost entirely abolished in the presence of Rab5a(S34N) and both proteins remain predominantly in the soma. (B) The Rab5a mutant does not abolish axonal or dendritic trafficking of palmitoylation-deficient GAD65, which is still present in rare axonal puncta (enlarged frame). (C) Axonal and dendritic targeting of the 24-60GAD65-GFP protein in the absence of Rab5a(S34N). (D) Axonal and dendritic targeting of the 24-60GAD65-GFP protein is not inhibited in the presence of Rab5a(S34N). Scale bars, 10  $\mu$ m.

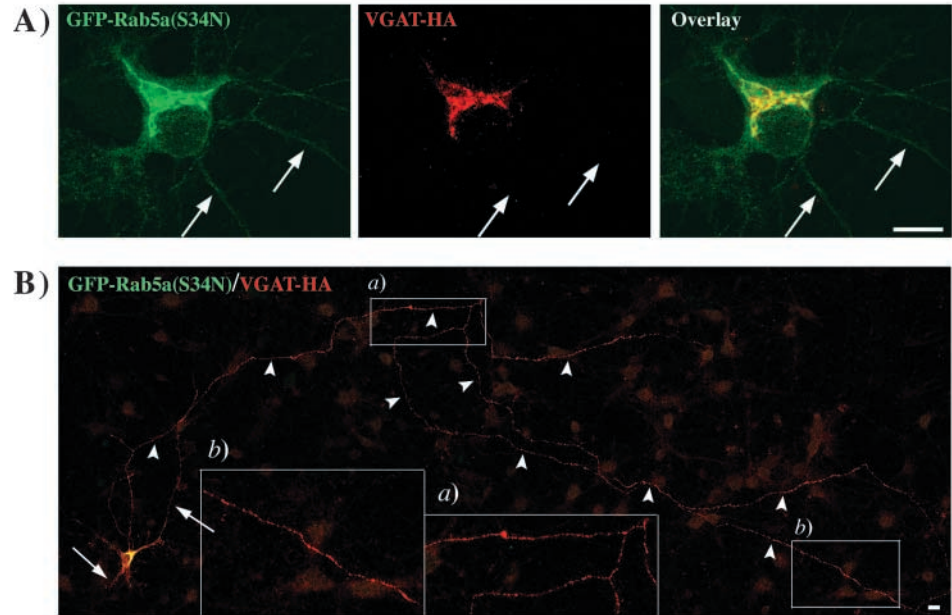


to be severely inhibited in the presence of the dominant negative mutant of Rab5a.

#### Rab5a(S34N) does not affect the trafficking of PSD-95 and delays, but does not block, the targeting of TfR

We next assessed whether somatodendritic Rab5a is involved in polarized trafficking of the transferrin receptor (TfR) and/or the post-synaptic density protein PSD-95. TfR is specifically targeted to dendrites (Burack et al., 2000) while PSD-95 appears to achieve its localization by specific retention in postsynaptic densities (El-Husseini et al., 2001). In 72-hour coexpression experiments, TfR-GFP was detected in dendritic puncta and in a perinuclear punctate pattern in the soma, where it colocalized with wt Rab5a (results not shown) and endogenous EEA1 (Fig. 10A). However, while images of Rab5a and EEA1 in somatodendritic puncta overlapped (Fig. 2A), TfR segregated from EEA1 (Fig. 10A, enlarged frame *a*) and Rab5a (not shown) in many puncta. Furthermore, TfR was detected in distal dendritic puncta that were devoid of Rab5a (results not shown) and EEA1 (Fig. 10A, enlarged frame *b*). The segregation of TfR and EEA1 was markedly different from the complete overlap of axonal and presynaptic images of Rab5a and each of the synaptic vesicle proteins described

above. The results suggest that TfR segregates from Rab5a/EEA1 within somatodendritic endosomes, whereas GAD65, VGAT and VAMP2 remain close to Rab5a in axonal endosomes. The segregation of TfR and Rab5a/EEA1 in tubular somatodendritic endosomal vesicles was reminiscent of that observed for Rab5 and another small GTP-binding protein, Rab4 (De Renzi et al., 2002), and might represent sorting into distinct endosomal compartments. TfR was absent from axons – consistent with its selective targeting to dendrites (Burack et al., 2000) – and showed no colocalization with endogenous GAD65 (results not shown). In the presence of HA-Rab5a(Q79L), TfR-GFP was absent from giant axonal endosomes and was exclusively detected in giant somatodendritic endosomes (Fig. 10B) as previously described (de Hoop et al., 1994). In 72-hour coexpression experiments with Rab5a(S34N), localization of TfR in somatodendritic endosomes was indiscernible from conditions without the mutant (Fig. 10C,D). However, in 24-hour coexpression experiments, TfR was primarily detected together with Rab5a(S34N) in the Golgi compartment and at the tip of dendrites (Fig. 10E, enlarged frames *a,b*) but not axons (Fig. 10E, enlarged frame *c*). Thus, although Rab5a(S34N) does not affect the polarized targeting of TfR it appears to delay its trafficking to somatodendritic endosomes. In sum, 1) TfR has



**Fig. 9.** The Rab5a(S34N) dominant negative mutant inhibits axonal trafficking and presynaptic clustering of VGAT. Confocal images of hippocampal neurons transfected with plasmids encoding VGAT-HA and GFP-tagged Rab5a(S34N). Neurons were double immunolabeled for HA and GFP. (A) A view of the soma reveals that VGAT-HA colocalizes with GFP-Rab5a(S34N) in the Golgi compartment. (B) A view of a neuron expressing VGAT-HA and GFP-Rab5a(S34N). The red color was enhanced to show the axon. VGAT shows a weak diffuse staining in axons and is not detected in presynaptic clusters in the proximal (enlarged frame *a*) or in the distal axon (enlarged frame *b*). Scale bars, 10  $\mu$ m.

an opposite polarization of GAD65 with regard to neuronal endosomes; 2) the disruption of polarized targeting of GAD65 in the presence of Rab5a(S34N) in 24-hour experiments is not observed for TfR; 3) TfR appears to segregate from Rab5a in somatodendritic endosomes; and, 4) Rab5a(S34N) appears to delay but not block the trafficking of TfR.

PSD-95-GFP did not colocalize with wt Rab5a or Rab5a(Q79L) in co-transfection experiments. Furthermore, Rab5a(S34N) did not affect targeting of PSD-95 to postsynaptic densities (see supplemental data Fig. S9, <http://jcs.biologists.org/supplemental/>). Thus an inhibitory effect of Rab5a(S34N) on trafficking of PSD-95 was not observed.

## Discussion

### A specific role of axonal Rab5a in trafficking to presynaptic clusters

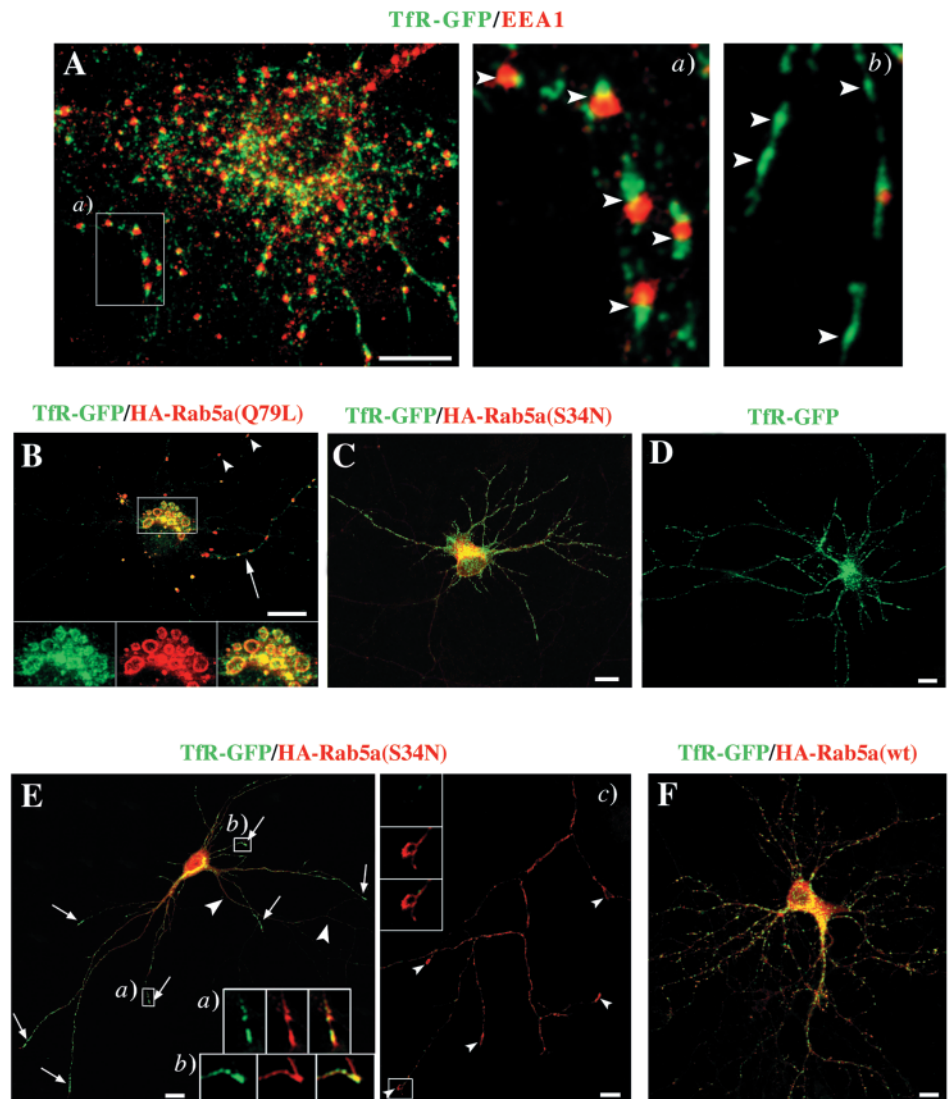
Neurons contain distinct axonal and somatodendritic early endosomal populations. While axonal endosomes might serve specialized neuronal functions such as the generation of synaptic vesicles (de Hoop et al., 1994; Takei et al., 1996), the TfR-enriched somatodendritic endosomes were suggested to serve common endosomal 'housekeeping' functions in both polar and non-polar cells, including the dissociation of ligands from receptors and the sorting of receptors to different destinations (Parton and Dotti, 1993; Wilson et al., 2000). Wilson et al. showed that the Rab5a effector molecule EEA1 is polarized in hippocampal neurons and other polarized cells (Wilson et al., 2000). In neurons, it is only present in somatodendritic endosomes containing Rab5a while axonal endosomes containing Rab5a are devoid of EEA1. This observation provided evidence that Rab5a in axons is associated with an axon-specific effector complex and suggested the possibility that Rab5a serves a distinct function in axonal and somatodendritic endosomes. The results presented here suggest that in axons, Rab5a controls an axon-specific trafficking pathway to presynaptic clusters that is

crucial for the targeting of some synaptic vesicle proteins. By contrast, while Rab5a(S34N) might cause a temporary delay in the trafficking of TfR, a crucial role of somatodendritic Rab5a in polarized sorting and trafficking of the dendritic proteins TfR and PSD-95 was not observed in our 72-hour experiments.

### Palmitoylation is required for post-Golgi sorting of GAD65 to the Rab5a-dependent axonal pathway

Newly synthesized cytosolic GAD65 undergoes hydrophobic modification(s) upstream of the palmitoylation signal, which result in a protein that targets to the cytosolic leaflet of Golgi membranes. Both wt and palmitoylation-deficient GAD65 target to Golgi membranes (Solimena et al., 1994; Kanaani et al., 2002) but targeting of the latter to presynaptic clusters is <5% of wt (Kanaani et al., 2002). The first evidence for a role of axonal Rab5a in the trafficking to presynaptic clusters came from experiments aimed at defining differences in the trafficking of wt and palmitoylation-deficient GAD65. In steady-state cultures of primary hippocampal neurons, palmitoylated GAD65 and wt axonal Rab5a colocalize in Golgi membranes and in presynaptic clusters. Palmitoylation-deficient GAD65 is also present in Golgi membranes but is absent from presynaptic clusters containing Rab5a. A dramatic difference between palmitoylation-deficient and wt GAD65 is the inability of the mutant to sort into the giant axonal vesicles which lack EEA1 and are induced by the constitutively active Rab5a(Q79L) protein. Furthermore, palmitoylation-deficient GAD65 is absent from endosomal profiles that accumulate in the tip of neurons in 24-hour expression experiments with the dominant negative Rab5a(S34N) mutant. Palmitoylated GAD65 is specifically sorted to axonal endosomes containing Rab5a(Q79L), but remains excluded from somatodendritic endosomes containing Rab5a and EEA1. Finally, the Rab5a(S34N) mutant, which remains in the GDP bound state, initially disrupts the preferential targeting of palmitoylated GAD65 to axons and then appears to block its post-Golgi targeting and

**Fig. 10.** Coexpression of TfR with wt and mutant Rab5a. Confocal images of hippocampal neurons expressing TfR-GFP with or without wt and mutant HA-Rab5a for 72 (A-D) or 24 hours (E-F). Neurons were double immunolabeled for GFP and either HA or endogenous EEA1. (A) A view of a neuron expressing TfR-GFP in somatodendritic endosomes where it colocalizes with endogenous EEA1. High magnification of proximal dendritic puncta (enlarged frame *a*), however, reveals segregation of TfR-GFP and EEA1 in tubular structures. A view of a distal dendrite (enlarged frame *b*) reveals puncta that contain TfR-GFP but are devoid of EEA1. (B) TfR-GFP colocalizes with HA-Rab5a(Q79L) in giant endosomal vesicles in the soma (enlarged frame) and in dendrites (arrows) but not in axons (arrowheads). (C) In 72-hour coexpression experiments with Rab5(S34N), TfR traffics to dendritic puncta and its trafficking is similar to conditions without the mutant as shown in D. (E) In 24-hour coexpression experiments, however, with Rab5a(S34N), TfR accumulates in the tips of dendrites (enlarged frames *a,b*) but not axons (enlarged frame *c*) and is largely absent from dendritic puncta. (F) For comparison, a view of a neuron coexpressing TfR and wt Rab5a for 24 hours shows trafficking to dendritic puncta. Scale bars, 10  $\mu$ m.



presynaptic clustering altogether. By contrast, Rab5a(S34N) does neither affect axonal targeting of the palmitoylation-deficient mutant nor the targeting of a second GAD65 mutant which targets to axonal puncta distinct from axonal endosomes and fails to reach presynaptic termini. Therefore, Rab5a(S34N) does not impose a general block on axonal trafficking but appears to specifically affect proteins that traffic to the axonal endosomal vesicles and presynaptic clusters. Our results suggest that palmitoylation labels GAD65 for an axonal trafficking pathway leading to presynaptic clusters, a trafficking pathway that intersects with axonal endosomes and is regulated by axonal Rab5a. ARF6, a small GTP-binding protein involved in recycling of endosomes, does not colocalize with GAD65 and a dominant negative mutant of ARF6 does not affect presynaptic clustering of GAD65 (our unpublished results). These results are consistent with a lack of intersection between the GAD65 trafficking pathway and a recycling-endosomal pathway, containing TfR, and controlled by ARF6. They provide further evidence for a specific role of early axonal endosomes and axonal Rab5a in the trafficking of GAD65. Because the GAD65-positive giant

early-endosomal vesicles that form in the presence of Rab5a(Q79L), line – starting at the soma – the entire axon, and because Rab5a plays a role in regulating the motility of early endosomes on microtubules (Nielsen et al., 1999), we propose that these vesicles transport newly synthesized GAD65 to axon termini.

The 24-60GAD65 protein lacks a Golgi-localization signal, anchors to membranes distinct from Golgi membranes, and targets to both axons and dendrites (Kanaani et al., 2002). This protein is not detected in the giant axonal endosomes induced by Rab5a(Q79L). Therefore, Golgi localization appears to be a pre-requisite for targeting of GAD65 to these endosomes. Palmitoylated GAD65 accumulates with Rab5a(S34N) in endocytic profiles observed in 24-hour coexpression experiments, while palmitoylation-deficient GAD65 remains confined to the Golgi compartment. We therefore propose that, palmitoylated and palmitoylation-deficient GAD65 diverge already in the Golgi compartment. The mechanism of such segregation appears to involve the association of the palmitoylated trafficking signal in GAD65 with cholesterol-rich microdomains in Golgi membranes (Kanaani et al., 2002

and unpublished results). It remains to be investigated whether Rab5a itself has a role in the sorting of palmitoylated GAD65 within Golgi membranes.

Palmitoylation is a reversible process. In the case of GAD65, palmitoylation controls a sorting event that is essential for an effective and selective transport to presynaptic clusters in axons. The functional consequences of such trafficking are probably significant because it positions the enzyme at the site of accumulation and secretion of its product GABA. Palmitoylation might therefore be controlled by signals that convey the inhibitory neuron's action potential and the requirements for rapid GABA secretion. The Golgi compartment might constitute a reservoir of GAD65 that can be sorted into the axonal trafficking pathway to presynaptic sites following palmitoylation. Similarly, it is conceivable that depalmitoylation might occur at different points of this pathway to release the protein from its high efficiency route to presynaptic clusters in axon termini.

Most newly synthesized membrane proteins traffic to early endosomes by a route that involves their transport from the TGN to the plasma membrane and a subsequent endocytosis step (reviewed in Keller and Simons, 1997). However, there is also evidence that proteins traffic to endosomal membranes directly from the TGN (Futter et al., 1995; Leitinger et al., 1995). We have not been able to observe accumulation of GAD65 in the plasma membrane in the presence of the Rab5a(S34N) mutant that blocks endocytosis. Moreover, while axonal transport and presynaptic clustering of GAD65 is blocked in the presence of a dynamin I mutant that blocks endocytosis, GAD65 accumulates in the Golgi compartment but is not detected in the plasma membrane (unpublished results). Thus it is possible that GAD65 traffics from the Golgi compartment to endosomes without going through the plasma membrane.

#### The Rab5a-dependent pathway is involved in trafficking of additional synaptic vesicle proteins to axon termini

Our results suggest that the Rab5a-dependent axonal trafficking pathway identified for GAD65 is not restricted to this protein. The synaptic vesicle proteins VGAT-HA and VAMP2-GFP both target to the giant axonal endosomes induced by Rab5a(Q79L). Furthermore, axonal trafficking of VGAT-HA, and VAMP2-GFP was blocked in the presence of the dominant negative Rab5a(S34N) mutant. The almost complete block in axonal transport of GAD65, VGAT and VAMP-2 imposed by Rab5a(S34N), suggest that a Rab5a controlled pathway is crucial for presynaptic trafficking of these proteins. It should be noticed, however, that this dominant negative mutant, possibly exerts a wider effect than blocking only a Rab5a mediated pathway. Therefore, it is conceivable that Rab5a(S34N) blocks pathways which are mediated by other Rab5 isoforms and/or other Rab proteins that bind to the same exchange factors as Rab5a.

#### Sorting to axonal endosomes

A question that also remains is, whether post-Golgi trafficking of GAD65 involves sorting to already formed Rab5a-positive/EEA1-negative axonal endosomes or the association with a less specialized precursor compartment before the

segregation of somatodendritic and axonal endosomes. In our experiments, most of the early endosomes in the soma contained both Rab5a and EEA1, and some contained TfR, VAMP2 and VGAT, but all were completely devoid of GAD65, suggesting that this protein does not accumulate in somatodendritic early or sorting endosomes. Furthermore, although a few Rab5a-positive/EEA1-negative endosomal puncta were detected in the soma of some neurons, we were unable to detect GAD65 in such vesicles except at the very base of the axon. In fact, high-magnification confocal analysis of GAD65-GFP in the soma of neurons only detected the protein in Golgi membranes. Thus our data seem to favor the possibility that GAD65 sorts exclusively to axonal endosomes. However, if non-polarized early endosomal compartments indeed exist in primary neurons, the presence of GAD65 in such compartments might be too short-lived for detection in steady-state experiments. Studies of the trafficking of the synaptic vesicle proteins and of axonal versus somatodendritic Rab5a by live cell imaging might shed more light on this issue.

Also unclear is, whether the Rab5a-positive/EEA1-negative axonal vesicles of endosomal origin are direct precursors of synaptic vesicles or whether they first fuse with the plasma membrane at axon termini prior to the formation of synaptic vesicles. There is evidence that synaptic vesicle proteins can be transported to axon termini by different transport organelles and only become assembled into synaptic vesicles at the synapse (Okada et al., 1995). The results presented here, suggest that at least a subfraction of synaptic vesicle proteins share a common Rab5a-positive/EEA1-negative vesicle to axon termini.

We thank G. Banker, R. Collins, R. Edwards, J. Rothman and P. Stahl for reagents, P. Walter for the use of the confocal microscope, H. Finlay, A. Aguilera-Moreno and C. Go for technical help, and M. Morado and L. Yu for help with references. Support by grants from Nora Eccles Treadwell Foundation (SB), National Institutes of Health (SB, DSB), The American Heart Association (DSB) and The Medical Research Council of Canada (AEE) is gratefully acknowledged.

#### References

- Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B. and Zerial, M. (1992). The small GTPase rab5a functions as a regulatory factor in the early endocytic pathway. *Cell* **70**, 715-728.
- Burack, M. A., Silverman, M. A. and Banker, G. (2000). The role of selective transport in neuronal protein sorting. *Neuron* **26**, 465-472.
- Chang, Y. C. and Gottlieb, D. I. (1988). Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J. Neurosci.* **7**, 2123-2130.
- Christgau, S., Aanstoot, H. J., Schierbeck, H., Begley, K., Tullin, S., Hejnaes, H. and Bækkeskov, S. (1992). Membrane anchoring of the autoantigen GAD<sub>65</sub> to microvesicles in pancreatic β-cells by palmitoylation in the N-terminal domain. *J. Cell Biol.* **118**, 309-320.
- Christoforidis, S., McBride, H. M., Burgoyne, R. D. and Zerial, M. (1999). The Rab effector EEA1 is a core component of endosome docking. *Nature* **397**, 621-624.
- de Hoop, M., Huber, L., Stenmark, H., Williamson, E., Zerial, M., Parton, R. G. and Dotti, C. G. (1994). The involvement of the small GTP-binding protein rab5a in neuronal endocytosis. *Neuron* **13**, 1-20.
- de Renzis, S., Sonnichsen, B. and Zerial, M. (2002). Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. *Nat. Cell Biol.* **4**, 124-133.
- Dotti, C. G. and Simons, K. (1990). Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* **62**, 63-72.

- El-Husseini, A. E., Craven, S. E., Brock, S. C. and Brecht, D. S. (2001). Polarized targeting of peripheral membrane proteins in neurons. *J. Biol. Chem.* **276**, 44984-44992.
- Futter, C. E., Conolly, C. N., Cutler, D. F. and Hopkins, C. R. (1995). Newly synthesized transferrin receptors can be detected in the endosome before they appear on the cell surface. *J. Biol. Chem.* **270**, 10999-11003.
- Hannah, M. J., Schmidt, A. A. and Huttner, W. B. (1999). Synaptic vesicle biogenesis. *Annu. Rev. Cell Dev. Biol.* **15**, 733-798.
- Kanaani, J., Lissin, D., Kash, S. F. and Baekkeskov, S. (1999). The hydrophilic isoform of glutamate decarboxylase, GAD67, is targeted to membranes and nerve terminals independent of dimerization with the hydrophobic membrane-anchored isoform, GAD65. *J. Biol. Chem.* **274**, 37200-37209.
- Kanaani, J., El-Husseini, A. E., Aguilera-Moreno, A., Diacovo, J. M., Brecht, D. and Baekkeskov, S. (2002). A combination of three distinct trafficking signals mediates axonal targeting and presynaptic clustering of GAD65. *J. Cell Biol.* **158**, 1229-1238.
- Kaether, C., Skehel, P. and Dotti, C. G. (2000). Axonal membrane proteins are transported in distinct carriers: A two-color video microscopy study in cultured hippocampal neurons. *Mol. Biol. Cell* **11**, 1213-1224.
- Keller, P. and Simons, K. (1997). Post-Golgi biosynthetic trafficking. *J. Cell Sci.* **110**, 3001-3009.
- Keller, P., Toomre, D. K., Diaz, E., White, J. and Simons, K. (2001). Multicolour imaging of post-Golgi sorting and trafficking in living cells. *Nature Cell Biol.* **3**, 140-149.
- Leitinger, B., Hille-Rehfeld, A. and Spiess, M. (1995). Biosynthetic transport of the asialoglycoprotein receptor H1 to the cell surface occurs via endosomes. *Proc. Natl. Acad. Sci.* **92**, 10109-10113.
- McIntire, S. L., Reimer, R. J., Schuske, K., Edwards, R. H. and Jorgensen, E. M. (1997). Identification and characterization of the vesicular GABA transporter. *Nature* **389**, 870-876.
- Miesenbock, G., De Angelis, D. and Rothman, J. E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394**, 192-195.
- Namchuk, M., Lindsay, L.-A., Turck, C. W., Kanaani, J. and Baekkeskov, S. (1997). Phosphorylation of serine residues 3,6,10, and 13 distinguishes membrane anchored from soluble glutamic acid decarboxylase 65 and is restricted to glutamic acid decarboxylase 65 $\alpha$ . *J. Biol. Chem.* **272**, 1548-1557.
- Nielsen, E., Severin, F., Backer, J. M., Hyman, A. A. and Zerial, M. (1999). Rab5 regulates motility of early endosomes on microtubules. *Nat. Cell Biol.* **1**, 376-382.
- Novick, P. and Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. *Curr. Opin. Cell Biol.* **9**, 496-504.
- Okada, Y., Yamazaki, H., Sekinealy, Y. and Hirokawa, N. (1995). The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde transport of synaptic vesicle precursors. *Cell* **81**, 769-780.
- Parton, R. G. and Dotti, C. G. (1993). Cell biology of neuronal endocytosis. *J. Neurosci. Res.* **36**, 1-9.
- Roberts, R. L., Barbieri, M. A., Pryse, K. M., Chua, M., Morisaki, J. J. and Stahl, P. D. (1999). Endosome fusion in living cells overexpressing GFP-rab5. *J. Cell Sci.* **112**, 3667-3675.
- Sampo, B., Kaech, S., Kunz S. and Banker G. (2003). Two distinct mechanisms target membrane proteins to the axonal surface. *Neuron* **37**, 611-624.
- Shi, Y., Veit, B. and Baekkeskov, S. (1994). Amino acid residues 24-31 but not palmitoylation of cysteines 30 and 45 are required for membrane anchoring of glutamic acid decarboxylase, GAD<sub>65</sub>. *J. Cell Biol.* **124**, 927-934.
- Solimena, M., Dirks, R., Jr, Radzynski, M., Mundigl, O. and De Camilli, P. (1994). A signal located within amino acids 1-27 of GAD65 is required for its targeting to the Golgi complex region. *J. Cell Biol.* **126**, 331-341.
- Stenmark, H., Parton, R. T., Steele-Mortimer, O., Lutcke, A., Gruenberg, J. and Zerial, M. (1994). Inhibition of Rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO J.* **13**, 1287-1296.
- Takei, K., Mundigl, O., Daniell, L. and De Camilli, P. (1996). The sv cycle: a single vesicle budding step involving clathrin and dynamin. *J. Cell Biol.* **133**, 1237-1250.
- Tienari, P. J., De Strooper, B., Ikonene, E., Simons, M., Weidemann, A., Czech, C., Hartmann, T., Ida, N., Multhaup, G., Masters, C. L. et al. (1996). The beta-amyloid domain is essential for axonal sorting of amyloid precursor protein. *EMBO J.* **15**, 5218-5229.
- Wilson, J. M., de Hoop, M., Zorzi, N., Toh, B.-H., Dotti, C. G. and Parton, R. G. (2000). EEA1, a tethering protein of the early sorting endosome, shows a polarized distribution in hippocampal neurons, epithelial cells, and fibroblasts. *Mol. Biol. Cell* **11**, 2657-2671.
- Winkler, B. and Mellman, I. (1999). Neuronal polarity: controlling the sorting and diffusion of membrane components. *Neuron* **23**, 637-640.
- Wucherpfennig, T., Wilsch-Bräuninger, M. and González-Gaitán, M. (2003). Role of *Drosophila* Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *J. Cell Biol.* **161**, 609-624.