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# A combination of three distinct trafficking signals mediates axonal targeting and presynaptic clustering of GAD65

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The signals involved in axonal trafficking and presynaptic clustering are poorly defined. Here we show that targeting of the  $\gamma$ -aminobutyric acid-synthesizing enzyme glutamate decarboxylase 65 (GAD65) to presynaptic clusters is mediated by its palmitoylated 60-aa NH<sub>2</sub>-terminal domain and that this region can target other soluble proteins and their associated partners to presynaptic termini. A Golgi localization signal in aa 1–23 followed by a membrane anchoring signal upstream of the palmitoylation motif are required for this process and mediate targeting of GAD65 to the cytosolic leaflet of Golgi membranes, an obligatory first step in axonal sorting. Palmitoylation of

a third trafficking signal downstream of the membrane anchoring signal is not required for Golgi targeting. However, palmitoylation of cysteines 30 and 45 is critical for post-Golgi trafficking of GAD65 to presynaptic sites and for its relative dendritic exclusion. Reduction of cellular cholesterol levels resulted in the inhibition of presynaptic clustering of palmitoylated GAD65, suggesting that the selective targeting of the protein to presynaptic termini is dependent on sorting to cholesterol-rich membrane microdomains. The palmitoylated NH<sub>2</sub>-terminal region of GAD65 is the first identified protein region that can target other proteins to presynaptic clusters.

## Introduction

Polarized sorting and efficient transport of proteins to presynaptic sites in axons and postsynaptic sites in dendrites is fundamental for neuronal function. Yet, the targeting signals and mechanisms involved in this process remain obscure (for review see Winckler and Mellman, 1999). Although trafficking signals that mediate selective targeting to dendritic membranes in neurons may have factors in common with signals involved in basolateral targeting in epithelial cells (Jareb and Banker, 1998), signals that mediate selective targeting to axons have yet to be identified. One proposed mechanism for polarized trafficking involves the segregation of axonal and dendritic proteins in the TGN and transport in distinct vesicles that traffick to either axons or dendrites (Bradke and Dotti, 1998). Analysis of vesicular transport of the transferrin receptor in live cells suggests that this protein

is selectively transported to the dendritic surface (Burack et al., 2000). In contrast, similar analyses were consistent with a mechanism of nonselective transport, but specific retention, of NgCAM in axons (Burack et al., 2000).

In this study, we have explored the trafficking signals that target the smaller isoform of the  $\gamma$ -aminobutyric acid (GABA)\*-synthesizing enzyme glutamate decarboxylase 65 (GAD65) to presynaptic termini in axons. GABA synthesized by GAD65 is required for fine tuning of GABA-ergic neurotransmission in response to a variety of environmental stimuli (Kash et al., 1997, 1999; Hensch et al., 1998). GAD65 is synthesized as a hydrophilic cytosolic protein, which undergoes hydrophobic posttranslational modifications in the NH<sub>2</sub>-terminal domain, resulting in anchoring to intracellular membranes (Christgau et al., 1991), including the Golgi compartment (Solimena et al., 1993, 1994). aa 1–83 of GAD65 were shown to mediate Golgi targeting of a soluble protein,  $\beta$  galactosidase, whereas an inter-










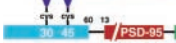
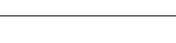
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Key words: palmitoylation; Golgi targeting; post-Golgi targeting; polarized sorting; neurons

\*Abbreviations used in this paper: GABA,  $\gamma$ -aminobutyric acid; GAD65, glutamate decarboxylase 65; GKAP, guanylate kinase-associated protein; GM130, Golgi matrix protein 130; MAP2, microtubule-associated protein 2; PSD, postsynaptic density; wt, wild type.

Figure 1. **Summary of constructs and key features.** Palmitoylation and membrane anchoring data were obtained in COS-7 cells. Targeting data are from primary hippocampal neurons.

CONSTRUCTS	KEY FEATURES	TARGETS TO				
		Presynaptic Clusters	Axons Diffuse	Postsynaptic Densities	Dendrites Diffuse	Golgi
	Wild-type GAD65 palmitoylated membrane anchored	Yes	No	No	No	Yes
	Wild-type GAD65-GFP palmitoylated membrane anchored	Yes	No	No	No	Yes
	GAD65(C30,45A)-GFP palmitoylation deficient membrane anchored	<5% of wt	Yes	No	Yes	Yes
	GAD65(24-31A)-GFP palmitoylation deficient soluble	No	Yes	No	Yes	No
	1-72GAD65-GFP palmitoylated membrane anchored	Yes	No	No	No	Yes
	1-72GAD65(C30,45A)-GFP palmitoylation deficient membrane anchored	No	Yes	No	Yes	Yes
	1-72GAD65(24-31A)-GFP palmitoylation deficient soluble	No	Yes	No	Yes	No
	24-60GAD65-GFP palmitoylated membrane anchored	No	No	No	No	No
	PSD-95-GFP palmitoylated membrane anchored	No	No	Yes	No	No
	PSD-95(C3,5S)-GFP palmitoylation deficient soluble	No	Yes	No	Yes	No
	1-60GAD65/Δ1-13PSD-95-GFP palmitoylated membrane anchored	Yes	No	No	No	Yes

change of aa 1–27 of GAD65 with aa 1–29 of a second isoform of glutamate decarboxylase, GAD67, mediated targeting of the latter to Golgi membranes (Solimena et al., 1994). However, because GAD67 appears to harbor intrinsic membrane anchoring properties (Kanaani et al., 1999), it is unclear whether the aa 1–27 region of GAD65 is sufficient for targeting of soluble proteins to Golgi membranes. Palmitoylation of cysteines 30 and 45 and phosphorylation of three of the serines 3, 6, 10, and 13 are exclusive properties of the membrane-anchored form of GAD65 (Christgau et al., 1992; Namchuk et al., 1997). However, neither modification is required for Golgi targeting or membrane association (Shi et al., 1994; Solimena et al., 1994; Namchuk et al., 1997). Instead, the results of Shi et al. (1994), suggest a critical role of aa 24–31, or a subset thereof, in membrane anchoring. These residues may comprise a signal sequence for attaching a putative membrane anchor. In addition to the Golgi compartment, GAD65 in pancreatic  $\beta$  cells occurs on the membrane of small vesicles, which are similar in size to synaptic-like microvesicles (Christgau et al., 1992). Immunofluorescence analysis of primary neuronal cultures shows that GAD65 targets to presynaptic termini, where it presumably resides on synaptic vesicles (Kanaani et al., 1999). The localization of GAD65 on the membrane of synaptic vesicles may be critical for rapid accumulation and secretion of GABA in response to environmental signals.

In this paper, we show that two separate signals are required for targeting of cytosolic GAD65 to Golgi membranes and that Golgi membranes constitute an essential sorting station and reservoir for GAD65 in route to axons. Although palmitoylation of a third trafficking signal in GAD65 is not required for targeting to the Golgi compartment, it is critical for trafficking of GAD65 from Golgi membranes to presynaptic sites.

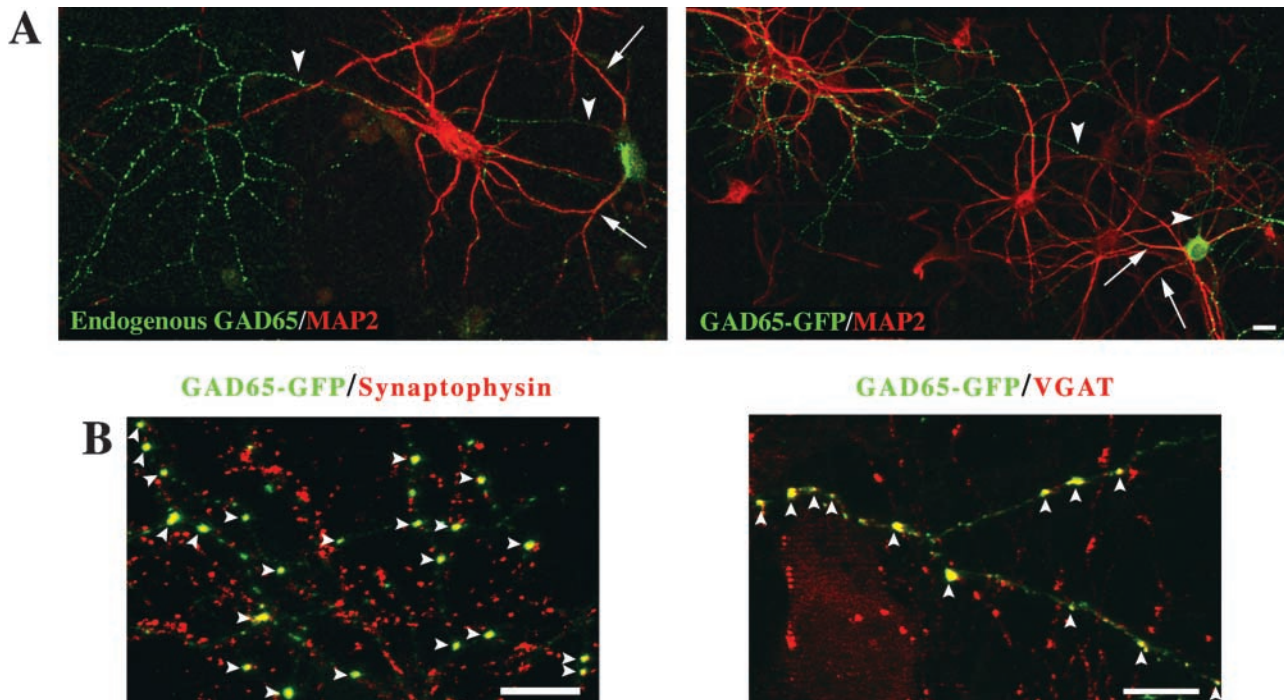
## Results

### GAD65 and GAD65–GFP are targeted to presynaptic clusters in hippocampal neurons

GFP chimeras were expressed in COS-7 cells, analyzed by Western blotting for expression as full-length proteins, and, by subcellular fractionation, followed by Western blotting for distribution between soluble and membrane fractions (Fig. 1). The subcellular targeting of endogenous GAD65 and transfected GAD65–GFP fusion proteins were analyzed by immunofluorescence of primary hippocampal neurons. GAD65 in hippocampal neurons (Fig. 2 A, left) localizes almost exclusively to presynaptic clusters in axons and to a perinuclear region in the cell body. Analysis of the GAD65–GFP chimera in transfected neurons revealed a localization that was similar to endogenous GAD65 (Fig. 2 A, right; Fig. 2 B). Thus, addition of GFP to the COOH terminus of GAD65 does not seem to affect its targeting and subcellular localization.

### Palmitoylation is essential for effective targeting of GAD65 to presynaptic clusters in axons and for relative exclusion from dendrites

To study the role of palmitoylation in axonal targeting and presynaptic clustering of GAD65, a palmitoylation-deficient mutant, GAD65(C30,45A), was expressed as a GFP chimera in primary hippocampal neurons. These analyses revealed that palmitoylation is critical for efficient targeting of GAD65 to presynaptic sites in neurons. The palmitoylation-deficient protein was concentrated in the perinuclear region and showed diffuse staining in both axons and dendrites, and presynaptic clustering was largely abolished (Figs. 3 and 4). In contrast, analysis of the GAD65(S3,6,10,13A)–GFP mutant revealed that loss of phosphorylation did not affect the targeting of GAD65 (Fig. 4 B). The average total number of puncta in axons per neuron immunostained for wild-

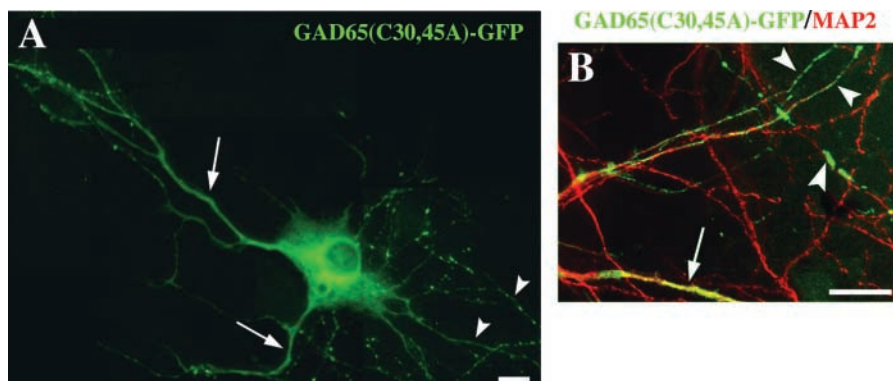


**Figure 2. Selective targeting of GAD65 and GAD65-GFP to presynaptic sites.** Confocal immunofluorescence analysis of endogenous GAD65 and transfected GAD65-GFP in cultured hippocampal neurons. (A) Endogenous GAD65, visualized by human monoclonal antibodies MICA 2, 3, and 6, and GAD65-GFP localize to a dense perinuclear region and to clusters in axons (arrowheads). The proteins are relatively excluded from dendrites (arrows) stained for endogenous MAP2 and are only detected in proximal dendrites, which are part of the cell body. (B) GAD65-GFP colocalizes with markers of presynaptic clusters, endogenous synaptophysin (all neurons), and vesicular GABA transporter (GABA-secreting inhibitory neurons) in axonal puncta revealing that they are presynaptic nerve terminals (arrowheads). Bars, 10  $\mu\text{m}$ .

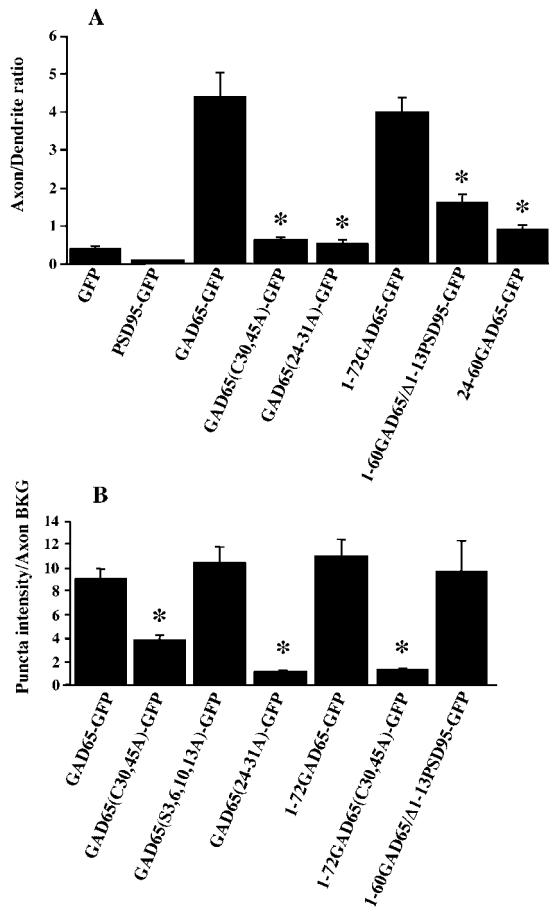
type (wt) GAD65-GFP was  $270 \pm 19$  (mean  $\pm$  SD,  $n = 6$ ). In comparison, the number of puncta in axons expressing the palmitoylation mutant in parallel cultures of similar density was  $48 \pm 12$  ( $n = 6$ ), or 18% of wild type. Even this small number of puncta was not all presynaptic axon terminals. Only 42% of puncta (61 of 145) expressing the palmitoylation-deficient protein contained synaptophysin compared with 94% (145 of 154) of the puncta expressing wt GAD65-GFP, bringing the number of presynaptic clusters containing the palmitoylation-deficient GAD65 to 8.2%. Furthermore, in clusters of the palmitoylation-deficient GAD65, the ratio of protein in puncta versus axon background was  $<50\%$  of that measured for wt GAD65-GFP

(Fig. 4 B), suggesting a low concentration of the palmitoylation mutant in such clusters. Thus, the total amount of the palmitoylation-deficient GAD65(C30,45A) protein in presynaptic clusters appears to be 3.6% of wt GAD65-GFP.

Culture of primary neurons in the presence of 10  $\mu\text{M}$  2-bromopalmitate, a palmitate analogue and inhibitor of palmitoylation, resulted in a distribution of wt GAD65-GFP similar to that of GAD65(C30,45A)-GFP. In this condition, wt GAD65 was mainly detected in the soma and dendrites and presynaptic clustering was largely abolished (Fig. S1 *i*, available at <http://www.jcb.org/cgi/content/full/jcb.200205053/DC1>). In comparison, treatment with 10  $\mu\text{M}$  palmitate had no effect on the targeting of GAD65-



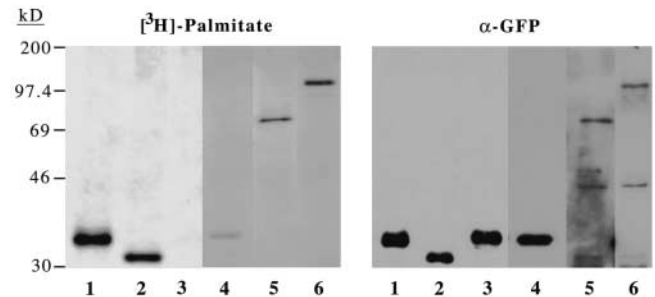
**Figure 3. Palmitoylation is required for presynaptic clustering of GAD65.** Immunofluorescence analysis of the palmitoylation-deficient mutant, GAD65(C30,45A)-GFP, in primary hippocampal neurons. (A) The palmitoylation-deficient mutant is targeted to both axons and dendrites, in addition to the perinuclear region. A small fraction is detected in axonal puncta. (B) Costaining of GAD65(C30,45A)-GFP with the dendritic marker MAP2 shows the lack of selective targeting to axons (arrowheads). The palmitoylation-deficient mutant is diffusely expressed in both dendrites (arrows) and axons, with a minor fraction detected in axonal puncta. Bars, 10  $\mu\text{m}$ .



**Figure 4. Quantitative analysis of axon/dendrite distribution and presynaptic clustering of GFP fusion proteins.** (A) Mean axon/dendrite ratios of targeting constructs. The A/D ratio of 1-72GAD65-GFP is not significantly different from wt GAD65-GFP. The A/D ratios of GAD65(C30,45A)-GFP, GAD65(24-31A)-GFP, 1-60GAD65/Δ1-13PSD-95-GFP, and 24-60GAD65-GFP are significantly different from wt GAD65-GFP (\* $P < 0.001$ ). The A/D ratio of 1-60GAD65/Δ1-13PSD-95-GFP is also significantly different from PSD-95-GFP ( $P < 0.001$ ). (B) Mean axonal puncta versus axonal background intensity of targeting constructs. The 1-72GAD65-GFP, 1-60GAD65/Δ1-13PSD-95-GFP, and GAD65(S3,6,10,13A)-GFP mutants are not significantly different from wt GAD65-GFP. The palmitoylation-deficient GAD65(C30,45A)-GFP and the soluble GAD65(24-31A)-GFP are significantly different from wt GAD65-GFP. Furthermore, 1-72GAD65(C30,45A)-GFP is significantly different from 1-72GAD65-GFP (\* $P < 0.001$ ).

GFP (Fig. S1 *ii*). In conclusion, palmitoylation is essential for relative dendritic exclusion and effective trafficking of GAD65 to presynaptic sites.

The NH<sub>2</sub>-terminal region of GAD65 has been shown to contain a membrane targeting signal upstream of the palmitoylated cysteines (Shi et al., 1994; Solimena et al., 1994). The GAD65(24-31A) mutation disrupted membrane association and yielded a soluble nonpalmitoylated protein in COS-7 cells (Fig. 1). This protein displayed a diffuse distribution in soma, dendrites, and axons, with no punctate staining (Fig. 4; Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200205053/DC1>). Thus membrane anchoring is a likely requirement for trafficking of GAD65 to presynaptic sites.

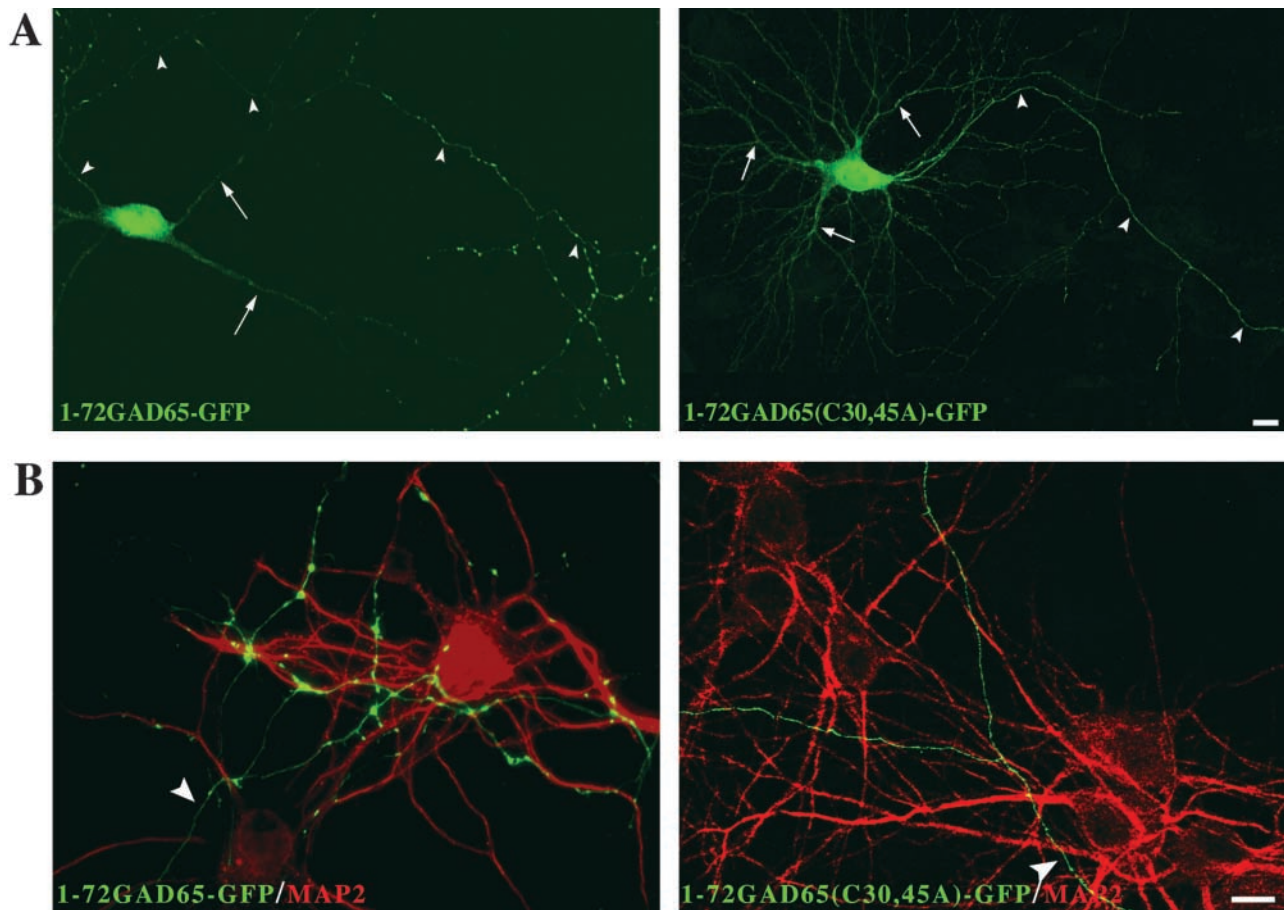


**Figure 5. Palmitoylation of GAD65-GFP fusion proteins.** Transfected COS-7 cells were metabolically labeled with [<sup>3</sup>H]palmitic acid. Immunoprecipitates with guinea pig anti-GFP were separated by SDS-PAGE and analyzed by fluorography (left) or immunoblotting using mouse monoclonal anti-GFP antibodies (right). The palmitoylation-deficient 1-72GAD65(C30,45A)-GFP (lane 3) and the 1-72GAD65(24-31A)-GFP mutant (lane 4) are expressed at similar levels as 1-72GAD65-GFP (lane 1) and 24-60GAD65-GFP (lane 2). But, only the latter two are robustly palmitoylated. The chimera carrying the palmitoylation motif of GAD65 in front of palmitoylation-deficient PSD-95 (1-60GAD65/Δ1-13PSD-95-GFP; lane 6) is palmitoylated to a similar level as wt GAD65-GFP (lane 5).

### The palmitoylated aa 1-60 region of GAD65 mediates presynaptic clustering of soluble proteins and their associated partners

To test whether the signals for presynaptic clustering reside in the palmitoylated NH<sub>2</sub>-terminal region of GAD65, we first generated a chimera of aa 1-72 coupled to GFP. This hybrid protein was palmitoylated (Fig. 5) and displayed presynaptic clustering and relative dendritic exclusion, similar to that of full-length GAD65-GFP (Figs. 4 and 6; Fig. S3 *i*, available at <http://www.jcb.org/cgi/content/full/jcb.200205053/DC1>). A C30,45A mutant of this protein, 1-72GAD65(C30,45A)-GFP, was not palmitoylated (Fig. 5). This protein displayed a diffuse expression pattern in axons and dendrites (Fig. 6). In contrast to the full-length palmitoylation mutant, none of the 1-72GAD65(C30,45A)-GFP protein was targeted to presynaptic clusters (Fig. 4 B; Fig. 6). Several conclusions can be made based on these results. First, the palmitoylated aa 1-72 region of GAD65 contains the necessary signals for presynaptic clustering of a soluble protein, GFP. Second, palmitoylation is essential for this process. Third, the signal(s) involved in the targeting of a small fraction of the full-length palmitoylation-deficient mutant to presynaptic clusters is missing in the short form of the palmitoylation-deficient mutant.

To further narrow down the targeting region, we assessed whether a slightly shorter version of the NH<sub>2</sub>-terminal region of GAD65, aa 1-60, can target a soluble mutant of the postsynaptic density (PSD) protein PSD-95 to presynaptic sites. A 1-60GAD65/Δ1-13PSD-95-GFP chimera was palmitoylated to a similar level as full-length GAD65-GFP (Fig. 5), targeted with a similar efficiency to presynaptic clusters in transfected neurons (Fig. 4 B; Fig. S3 *ii*), but had a higher background in proximal dendrites and a lower A/D ratio than GAD65-GFP (Fig. 4 A; Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200205053/DC1>). Expression of the 1-60GAD65/Δ1-13PSD-95-GFP chimera



**Figure 6. The signals for targeting of GAD65 to presynaptic clusters reside in aa 1–72 and palmitoylation of cysteines 30 and 45 is critical for this process.** Confocal analysis of hippocampal neurons transfected with either 1–72GAD65–GFP or 1–72GAD65(C30,45A)–GFP and single immunolabeled for GFP or double immunolabeled for GFP and MAP2. (A) A chimera of the palmitoylated aa 1–72 NH<sub>2</sub>-terminal domain of GAD65 and GFP is targeted to presynaptic clusters in axons (arrowheads) and relatively excluded from dendrites (arrows), similar to wt GAD65. In contrast, the palmitoylation-deficient 1–72GAD65(C30,45A)–GFP protein is detected in both axons and dendrites and there is no axonal clustering. (B) The palmitoylated 1–72GAD65–GFP chimera targets to axonal puncta that coincide with synapsing dendrites, identified by MAP2 staining. In contrast, palmitoylation-deficient 1–72GAD65(C30,45A)–GFP shows a diffuse staining in axons (arrowhead) and does not target to axonal puncta. Bars, 10  $\mu$ m.

also resulted in presynaptic clustering of endogenous guanylate kinase-associated protein (GKAP) (Fig. S4), a protein that normally associates with PSD-95 at postsynaptic densities (Kim et al., 1997). The results show that the aa 1–60 region of GAD65 harbors the necessary signals to direct the  $\Delta$ 1–13PSD-95 mutant and an endogenous protein partner to presynaptic sites, but that dendritic exclusion appears less efficient than for 1–72GAD65–GFP. This may be the result of a residual dendritic signal in  $\Delta$ 1–13PSD-95 and/or a partial role of aa 61–72 in dendritic exclusion of GAD65.

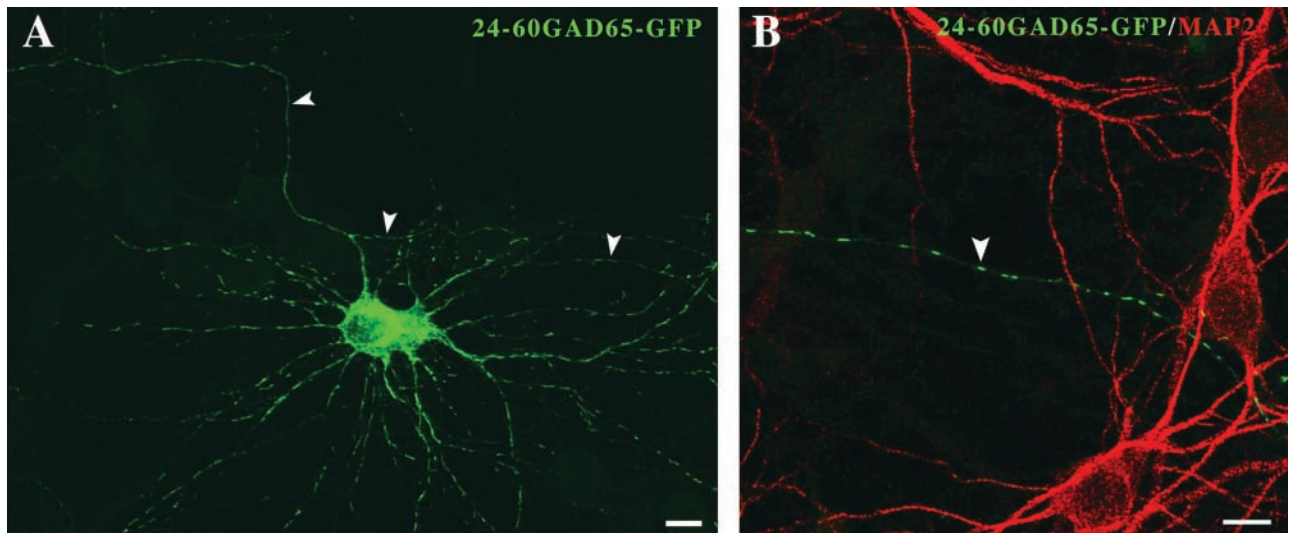
#### aa 1–23 in GAD65 contain a Golgi targeting signal and are required for presynaptic clustering

Neither palmitoylation nor the first 23 aa of GAD65 are required for membrane anchoring (Christgau et al., 1992; Shi et al., 1994), whereas aa 24–31, or a subset of this region, may harbor the putative membrane anchor (Shi et al., 1994). We tested whether the first 23 aa of the aa 1–60 region are required for targeting to presynaptic clusters by generating a chimera of aa 24–60 of GAD65 and GFP (24–60GAD65–GFP). The 24–60GAD65–GFP protein was

palmitoylated (Fig. 5) and membrane anchored in COS-7 cells (Fig. 1). In primary hippocampal neurons, the 24–60GAD65–GFP protein was detected in a punctate pattern in the soma and along both dendrites and axons, but completely failed to reach presynaptic clusters (Fig. 7; Fig. S3 *iii*). Thus, the first 23 aa are not required for membrane anchoring, but are indispensable for the targeting of GAD65 to presynaptic clusters.

Solimena et al. (1994) have shown that GAD65 localizes to Golgi membranes and that palmitoylation is not required for Golgi targeting. We performed detailed colocalization studies of GAD65 and the palmitoylation-deficient mutant in primary neurons and in COS-7 cells and confirmed this observation (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200205053/DC1>; unpublished data).

The 1–72GAD65–GFP, 1–72GAD65(C30,45A)–GFP, and 1–60GAD65/ $\Delta$ 1–13PSD-95–GFP proteins were also highly concentrated in a perinuclear region where they colocalized with Golgi matrix protein 130 (GM130) (Fig. 8; Fig. S5). The immunolabeled 24–60GAD65–GFP protein, however, was detected in a punctate pattern throughout the



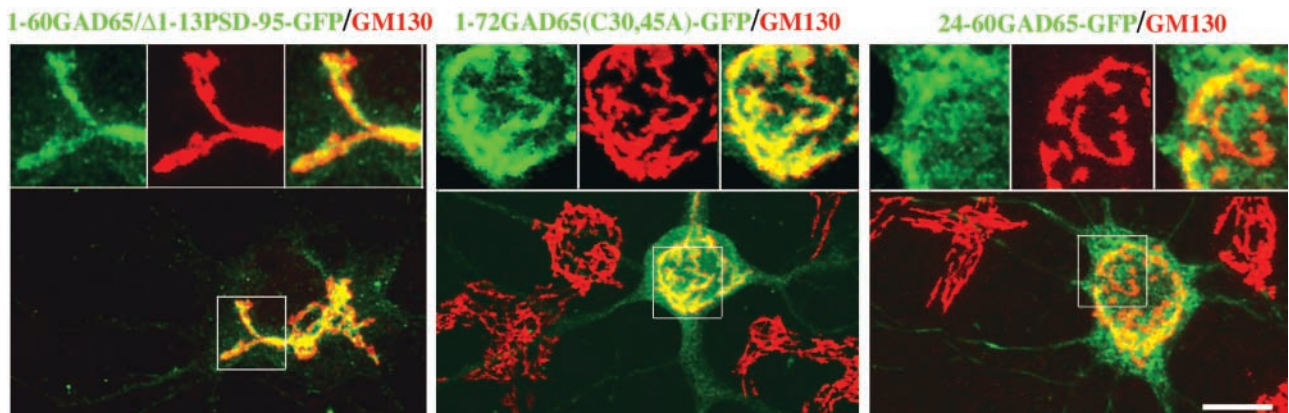
**Figure 7. aa 1–23 contain a critical targeting signal for presynaptic clustering.** Confocal images of hippocampal neurons expressing the 24–60GAD65–GFP protein and double immunolabeled for GFP and MAP2. (A) The 24–60GAD65–GFP protein is distributed in a punctate pattern in both dendrites and axons (arrowheads). (B) The axonal puncta do not coincide with synapsing dendrites from neighboring neurons. Bars, 10  $\mu$ m.

cytosol and did not concentrate in a perinuclear region defined by GM130 (Fig. 8). Thus, the 24–60GAD65–GFP protein mainly targets to membranes distinct from the Golgi compartment. These results suggest that membrane association and Golgi targeting are mediated by separate signals and that the intact aa 1–23 region is critical for the latter. Furthermore, the failure of the 24–60GAD65–GFP protein to target to presynaptic termini suggests that the Golgi compartment is an obligatory sorting station in route to axons.

#### Reduction in cellular cholesterol levels impairs specific targeting of wt GAD65 to presynaptic clusters

Sphingolipid–cholesterol-enriched lipid rafts form in the TGN (Simons and Wandinger-Ness, 1990) and some proteins may associate into rafts to achieve axonal localization (Ledesma et al., 1998). Palmitoylation promotes partition-

ing of proteins into rafts (Melkonian et al., 1999). To assess whether trafficking of GAD65 is sensitive to cellular levels of cholesterol, we analyzed the effect of cholesterol depletion on its targeting to presynaptic clusters. Neurons were treated with lovastatin in the presence of low amounts of mevalonate, to inhibit cholesterol biosynthesis, and were incubated with methyl- $\beta$ -cyclodextrin to extract cholesterol from plasma membranes. In initial experiments, conditions of methyl- $\beta$ -cyclodextrin treatment were established that resulted in maximum reduction of neuronal cholesterol levels without affecting the viability or integrity of neurons. Incubation of neurons in 5 mM methyl- $\beta$ -cyclodextrin for 20 min resulted in an  $\sim$ 30% reduction of cholesterol levels in the soma and  $\sim$ 60% reduction in neuronal processes. Fig. 9 shows the effect of cholesterol depletion on the distribution of GAD65 in axons. As described above, at normal levels of



**Figure 8. The aa 1–23 region is required for Golgi targeting of GAD65.** Confocal images of hippocampal neurons expressing mutant GAD65–GFP and double immunolabeled for GFP and the Golgi marker protein GM130. The palmitoylated 1–60GAD65/ $\Delta$ 1–13PSD-95–GFP and the palmitoylation-deficient 1–72GAD65(C30,45A)–GFP proteins primarily concentrate in the Golgi compartment in the soma of primary neurons, as revealed by colocalization with the GM130 protein. In contrast, the palmitoylated 24–60GAD65–GFP protein displays a dispersed punctate pattern throughout the soma and does not concentrate in the Golgi compartment. Bar, 10  $\mu$ m.

cholesterol, >90% of GAD65 in axons was detected in presynaptic clusters, identified as points of contact with synapsing dendrites (Fig. 9 A) or by costaining with synaptophysin (Fig. 2; unpublished data). The protein was virtually absent from axonal stretches that are devoid of dendritic contact (Figs. 2; Fig. 9 A). Cholesterol depletion, however, resulted in an even distribution of GAD65 in puncta along the entire axon (Fig. 9 B). Although an occasional puncta was located at a point of contact with a synapsing dendrite, there was no preferential localization of GAD65 to presynaptic clusters. Instead, the majority of GAD65-positive puncta were devoid of synapsing dendrites (Fig. 9 B) and did not contain synaptophysin (unpublished data). Thus, in conditions of cholesterol depletion, the exclusive targeting of GAD65 to presynaptic termini in axons is severely impaired. For comparison, trafficking of synaptophysin to presynaptic clusters, trafficking of the 24–60GAD65–GFP mutant to axonal and dendritic puncta, and trafficking of palmitoylation-deficient GAD65 to rare axonal puncta were similar in normal and cholesterol depletion conditions (unpublished data), suggesting that neuronal trafficking was not generally impaired by cholesterol depletion.

## Discussion

### Palmitoylation is essential for presynaptic clustering of GAD65 and the palmitoylated NH<sub>2</sub>-terminal aa 1–60 domain can direct other soluble proteins to this location

Palmitoylation is a mechanism for reversible localization of cytosolic and membrane proteins to a particular membrane environment (for review see Resh, 1999). The palmitoyl thioester group is not a targeting signal in and of itself, but rather contributes to a targeting function in the larger context of the specific aa sequence it resides within. In this paper, we show that dual palmitoylation of cysteines 30 and 45 in the GABA-synthesizing enzyme GAD65 provides a targeting signal that mediates selective targeting to presynaptic clusters. Palmitoylation is essential for the relative dendritic exclusion of GAD65 and for efficient trafficking from the Golgi compartment to presynaptic sites. The palmitoylated NH<sub>2</sub>-terminal domain of GAD65 contains all the necessary signals to mediate presynaptic targeting of GFP and of a soluble mutant of the postsynaptic protein PSD-95 along with its associated partner GKAP. A distinct closely spaced dual palmitoylation motif (cysteines 3 and 4) is essential for the axonal targeting of the growth cone protein GAP-43. However, a chimera of the palmitoylation domain of GAP-43 and the soluble mutant of PSD-95 is abundant in dendrites, displays only a slight increase in axonal targeting, and is not present in presynaptic clusters (El-Husseini et al., 2001). Thus, the palmitoylated NH<sub>2</sub>-terminal domain of GAD65 is the first identified protein region that selectively mediates sorting of other proteins and their partners to presynaptic clusters in axons.

### Golgi targeting is mediated by two trafficking signals distinct from the palmitoylation motif and is required, but not sufficient, for presynaptic clustering

A Golgi targeting signal resides in aa 1–23, and a lack of this signal results in a protein, 24–60GAD65–GFP, that anchors

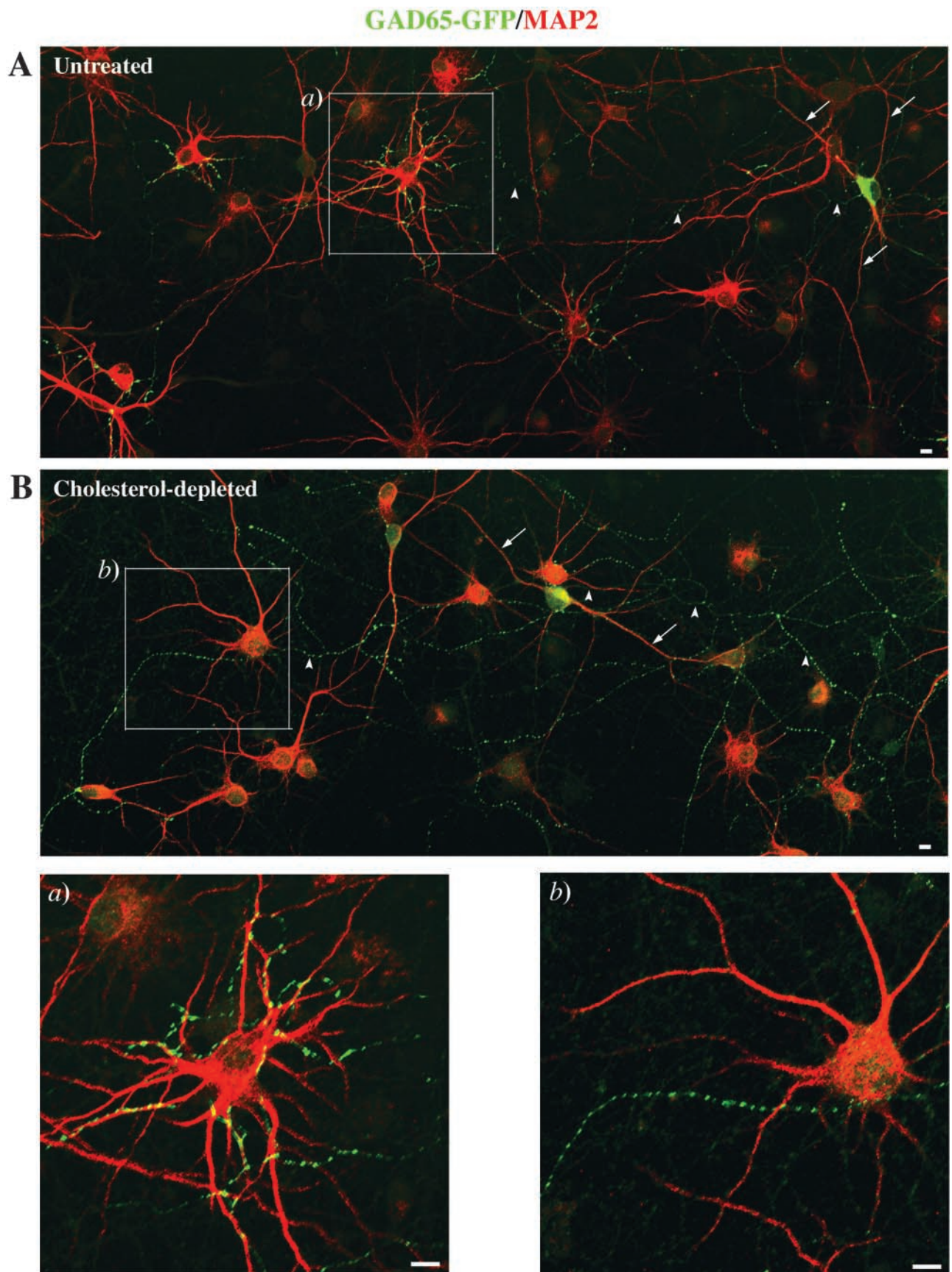
to intracellular membranes distinct from the Golgi compartment and fails to target to axon termini. Loss of the membrane anchoring signal yields a protein, GAD65(24–31A)–GFP, that is soluble and distributed equally throughout the neuron. Finally, loss of palmitoylation of cysteines 30 and 45 results in a protein that is membrane anchored and targets to the Golgi compartment but fails to enter a post-Golgi trafficking pathway for efficient and selective targeting to presynaptic termini. The targeting of the palmitoylation-deficient mutant to Golgi membranes separates Golgi localization from axonal transport in that only the latter demonstrably requires palmitoylation. Although palmitoylation can function as an essential part of a Golgi localization signal in some proteins (Lutjens et al., 2000), a lack of such a role is not without precedent; palmitoylation of lymphoma pro-protein convertase is not essential for its TGN localization (van de Loo et al., 2000), and dual palmitoylation of H-ras is not required for its Golgi targeting but rather for exit from the Golgi compartment and trafficking to the plasma membrane (Apolloni et al., 2000). These results demonstrate that the aa context of a palmitoylated trafficking signal, and/or the combination with other trafficking signals, is a critical parameter for determining membrane destination.

Anchoring to the cytosolic leaflet of Golgi membranes appears to provide a mechanism by which a soluble protein like GAD65 can gain access to a major sorting compartment for polarized proteins (Keller et al., 2001) and subsequently enter a post-Golgi trafficking pathway to axon termini, which requires membrane association. The 24–60GAD65–GFP protein does not localize to the Golgi compartment and yet is palmitoylated in COS-7 cells. It is therefore possible that newly synthesized GAD65 first anchors to the cytoplasmic leaflet of pre-Golgi membranes, possibly the ER or the ER–Golgi intermediate compartment, and that palmitoylation precedes trafficking to and accumulation in the Golgi compartment. The lack of a Golgi targeting signal in the 24–60GAD65–GFP protein would result in a palmitoylated protein that diverges from wt GAD65 in pre-Golgi membranes and bypasses the Golgi compartment. There is evidence to suggest the presence of a palmitoyl transferase in the ER. Thus, newly synthesized cytosolic H-ras undergoes farnesylation and carboxymethylation to become anchored to the cytoplasmic leaflet of the ER (Choy et al., 1999; Apolloni et al., 2000), where it appears to undergo palmitoylation before its trafficking to the Golgi compartment (Apolloni et al., 2000). An alternative possibility is that the palmitoyl transferase responsible for palmitoylation of cysteines 30 and 45 of GAD65 is in more than one location and that newly synthesized 24–60GAD65–GFP and wt GAD65–GFP undergo palmitoylation in two distinct membrane compartments.

### How does palmitoylation control trafficking of GAD65?

The results of cholesterol depletion experiments suggest that trafficking of palmitoylated GAD65 to presynaptic clusters is sensitive to cellular cholesterol levels. GAD65 is soluble in mild nonionic detergents and does not share the detergent insolubility of many proteins that stably associate with lipid rafts (Brown and Rose, 1992; Pralle et al., 2000). However, it was recently shown that palmitoylated H-ras has a revers-





**Figure 9. Cholesterol depletion results in impairment of presynaptic clustering of GAD65–GFP.** Confocal analysis of primary hippocampal neurons transfected with wt GAD65–GFP and cultured in either standard conditions (A) or a condition of cholesterol depletion (B). Cells were double immunolabeled for GFP and MAP2. (A) At normal cholesterol levels, GAD65 in axons is exclusively targeted to presynaptic clusters,

ible interaction with lipid rafts, which is not associated with detergent insolubility (Prior et al., 2001) and may represent a low-affinity mechanism afforded to other palmitoylated proteins. We propose that palmitoylation of GAD65 mediates its attachment to specialized membrane microdomains in the TGN, resulting in lateral segregation from the non-palmitoylated protein before the formation of transport vesicles. In a separate study, we have shown that palmitoylation of GAD65 regulates its entry into a post-Golgi trafficking pathway to axon termini, which involves early endosomes and Rab5a and is shared with several synaptic vesicle proteins (unpublished data). Thus, the trafficking of GAD65 appears to involve sorting steps in both the Golgi compartment and in early endosomes.

## Materials and methods

### Antibodies

The following antibodies were used in immunofluorescence experiments: rabbit polyclonal antibodies to synaptophysin (Zymed Laboratories), vesicular GABA transporter (McIntire et al., 1997; a gift from Dr. Robert Edwards, University of California San Francisco [UCSF]), and GKAP (Kim et al., 1997; a gift from Dr. Morgan Sheng, Massachusetts Institute of Technology, Cambridge, MA); mouse monoclonal antibodies to microtubule-associated protein 2 (MAP2), GM130 (Transduction Laboratories), and GFP (BABCO); and a guinea pig antibody raised to purified GFP-GST and affinity purified on a GFP-GST column. The GAD65-specific human monoclonal antibodies MICA 2, 3, and 6, derived from islet cell antibody-positive patients, were a gift from Dr. Wiltrud Richter (University of Heidelberg, Heidelberg, Germany) (Richter et al., 1993). Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories and Alexa Fluor®488-conjugated secondary antibodies were from Molecular Probes.

### DNA construction

All constructs used in this study, except GAD65(S3,6,10,13A), are shown in Fig. 1. Chimeras containing EGFP at the COOH-terminal end of either wt human GAD65, GAD65(S3,6,10,13A), or GAD65(24–31A) were generated by PCR and subcloned into the KpnI-BamHI sites in a pEGFP-N3 vector (CLONTECH Laboratories, Inc.). wt rat GAD65 and a palmitoylation-deficient mutant, GAD65(C30,45A), were generated by PCR and subcloned into the HindIII-KpnI sites in frame with and in front of EGFP in pEGFP-N3. The plasmids encoding wt and mutant GAD65 that served as templates in PCR reactions were described previously (Shi et al., 1994; Namchuk et al., 1997). wt PSD-95-GFP and PSD-95(C3,5S)-GFP in pGW1 were previously described (Craven et al., 1999). Fusions of aa 24–60 of wt GAD65, aa 1–72 of wt GAD65, the palmitoylation-deficient 1–72GAD65(C30,45A), or the soluble 1–72GAD65(24–31A) with the NH<sub>2</sub> terminus of EGFP were performed by PCR using primers encoding the 5' and 3' ends of aa 24–60 of wt GAD65 or aa 1–72 of wt GAD65, or the mutant forms, and the restriction sites HindIII-KpnI. Amplified fragments were digested and subcloned into the HindIII-KpnI sites of pEGFP-N3. To generate a chimera of the palmitoylated NH<sub>2</sub>-terminal domain of GAD65 and a palmitoylation-deficient PSD-95, a construct was generated by PCR using primers encoding the 5' and 3' ends of aa 1–60 of GAD65 and HindIII-KpnI restriction sites. The amplified fragments were digested and subcloned into GW1 PSD-95-GFP at a HindIII site upstream of the starter methionine and a silent KpnI site at aa 13 of PSD-95.

### COS-7 cell experiments

Culture, transfection, and labeling with [<sup>3</sup>H]palmitic acid of COS-7 cells were performed as described previously (Christgau et al., 1992; Shi et al., 1994; Namchuk et al., 1997). Cells were extracted in hypotonic Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM 2-aminoethyl-isothiuronium bromide, 0.2 mM pyridoxal 5'-phosphate, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-114). The 100,000 g supernatant was

immunoprecipitated with guinea pig anti-GFP antibodies and immunocomplexes were isolated and processed for SDS-PAGE followed by fluorography as previously described (Namchuk et al., 1997). For Western blotting, resolved proteins were electroblotted onto Protran BA nitrocellulose transfer membranes (Applied Scientific), probed with a primary antibody against GFP (monoclonal mouse; BABCO) and HRP-conjugated secondary antibodies (Amersham Biosciences), and visualized by ECL reagent (Amersham Biosciences).

### Hippocampal neurons

Primary hippocampal neurons were prepared from E18/E19 rat brains as described by Craven et al. (1999). Neurons were transfected at day in vitro (DIV) 6–7 by lipid-mediated gene transfer using Effectene transfection reagent according to the manufacturer's protocol (QIAGEN). After 3–5 h of incubation at 37°C, the transfection solution was replaced with a 50:50 solution of fresh/conditioned medium (replacement medium). Neurons were fixed 96 h after transfection. For experiments with 2-bromopalmitate, neurons were washed twice in replacement medium and then cultured in the same medium containing 10 μM of either palmitate or 2-bromopalmitate. Cells were fixed 48 h after transfection. Cholesterol depletion experiments and staining of neurons with filipin were performed essentially as described by Simons et al. (1998). Transfected neurons were cultured in replacement medium containing 4 μM lovastatin (A.G. Scientific, Inc.) and 0.25 mM mevalonate (Sigma-Aldrich) for 4 d. Cells were incubated for 20 min with 5 mM methyl-β-cyclodextrin (Sigma-Aldrich) in fresh neurobasal medium, washed twice with the same medium, and fixed. For analyses of cholesterol levels, fixed neurons were incubated with filipin III (Cayman Chemical) at 125 μg/ml in PBS.

### Immunofluorescence analyses

For indirect immunofluorescence, neuronal cultures were fixed in either 2% paraformaldehyde in PBS, pH 7.4, or methanol (–20°C) (staining for synaptophysin and GKAP). COS-7 cells were fixed 18–24 h after transfection with 2% paraformaldehyde. A guinea pig anti-GFP antibody (CLONTECH Laboratories, Inc.) was used as a primary antibody to enhance the signal of GFP chimeras in neuronal transfections. After incubation with primary and secondary antibodies, fluorescent images were obtained using either a ZEISS inverted microscope or a Leica TCS NT laser scanning confocal microscope with a krypton-argon laser.

### Quantitative measurement of polarized protein expression

Quantification of polarized protein sorting was performed on 5–20 neurons from two to three independent transfections. Images of neurons were acquired with a CCD digital camera and quantitated using Metamorph imaging software (Universal Imaging Corp.) (El-Husseini et al., 2001). The degree of presynaptic clustering or polarized expression of GAD65 was determined by calculating the average pixel intensity in the axon, axon puncta, and dendrites. The average pixel intensity was obtained by tracing five representative sections of both dendrites and axons, beginning at least 40 microns from the cell body. For constructs present in presynaptic clusters, lines were drawn through the axons including puncta. These measurements were averaged and used to calculate a ratio of axonal intensity (with or without puncta) versus dendritic intensity or axonal puncta intensity versus axonal background to compare distribution of GAD65-GFP and other constructs. These ratios are representative of the relative amounts of protein present in axons versus dendrites and in presynaptic clusters versus diffuse in axons, respectively. Results were analyzed by a *t* test using a two-tailed distribution and two-sample equal variance.

### Quantitative measurement of cholesterol levels

Fluorescence images of neurons incubated in either normal conditions or in cholesterol-depleting conditions and then fixed and incubated with the cholesterol-binding fluorescent antibiotic filipin III were captured on an Olympus IX-70 fluorescence microscope. Filipin III-stained cholesterol was excited with light at 365–395 nm. Fluorescence emissions at 425–465 nm were collected using a CCD digital camera. The amounts of background-subtracted fluorescence were determined for the perinuclear area in the soma and for areas in the neurites in each neuron and quantitated using Metamorph imaging software. Data collection parameters were maintained constant for all images and amounts of fluorescence from 50

identified as points of contact with synapsing dendrites stained for MAP2. (B) Reduction of cellular cholesterol levels impairs the specific trafficking of GAD65 to presynaptic termini and results in targeting of the protein to axonal puncta that are distributed evenly along the entire axon, without a preferential localization at synapses. Bars, 10 μm.

neurons for each condition were averaged. Statistically significant differences were determined using a nonpaired, two-tailed *t* test.

### Online supplemental material

The supplemental figures (Figs. S1–S5) for this article are available at <http://www.jcb.org/cgi/content/full/jcb.200205053/DC1>. These figures show confocal analysis of primary hippocampal neurons transfected with (a) wt GAD65–GFP and cultured in the presence of either 10 mM 2-bromopalmitate or 10 mM palmitate (Fig. S1); (b) GAD65(24–31A)–GFP (Fig. S2); (c) 1–72GAD65–GFP, 1–60GAD65/D1–13PSD-95–GFP, or 24–60GAD65–GFP and immunolabeled for GFP and endogenous synaptophysin (Fig. S3); (d) the 1–60GAD65/D1–13PSD-95–GFP chimera and double immunolabeled for GFP and GKAP (Fig. S4); and (e) wt or mutant GAD65–GFP and double immunolabeled for GFP and the Golgi marker protein GM130 (Fig. S5).

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