

A Signal Located within Amino Acids 1-27 of GAD65 Is Required for Its Targeting to the Golgi Complex Region

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Abstract. The mechanisms involved in the targeting of proteins to different cytosolic compartments are still largely unknown. In this study we have investigated the targeting signal of the 65-kD isoform of glutamic acid decarboxylase (GAD65), a major autoantigen in two autoimmune diseases: Stiff-Man syndrome and insulin-dependent diabetes mellitus. GAD65 is expressed in neurons and in pancreatic β -cells, where it is concentrated in the Golgi complex region and in proximity to GABA-containing vesicles. GAD65, but not the similar isoform GAD67 which has a more diffuse cytosolic distribution, is palmitoylated within its first 100 amino acids (a.a.). We have previously demonstrated that the domain corresponding to a.a. 1-83 of GAD65 is required for the targeting of GAD65 to the Golgi complex region. Here we show that this domain is sufficient to target an unrelated protein,

β -galactosidase, to the same region. Site-directed mutagenesis of all the putative acceptor sites for thio-palmitoylation within this domain did not abolish targeting of GAD65 to the Golgi complex region. The replacement of a.a. 1-29 of GAD67 with the corresponding a.a. 1-27 of GAD65 was sufficient to target the otherwise soluble GAD67 to the Golgi complex region. Conversely, the replacement of a.a. 1-27 of GAD65 with a.a. 1-29 of GAD67 resulted in a GAD65 protein that had a diffuse cytosolic distribution and was primarily hydrophilic, suggesting that targeting to the Golgi complex region is required for palmitoylation of GAD65. We propose that the domain corresponding to a.a. 1-27 of GAD65, contains a signal required for the targeting of GAD65 to the Golgi complex region.

THE identification of the signals responsible for the sorting of proteins to distinct intracellular compartments is an important step towards the elucidation of the complex architecture of the cell. While signals responsible for the targeting of proteins from the cytosolic compartment into membranous organelles have been extensively investigated, little is known about the targeting signals of cytosolic peripheral membrane proteins and cytosolic matrix proteins. Fatty acylation, including palmitoylation on cysteine residues, is one of the mechanisms involved in the targeting of cytosolic proteins to membranes (Chow et al., 1992). Palmitoylation is thought to mediate membrane association of several neuronal proteins which are enriched in axons and growth cones. These include GAP-43 (growth cone-associated protein of 43 kD) (Skene and Virag; 1989; Liu et al., 1991), a protein which plays a role in axon growth (Skene, 1989), SNAP-25 (synaptosomal-associated protein

of 25 kD) (Hess et al., 1992), a plasmalemma-associated protein involved in exocytosis from neurons (Oyler et al., 1989; Söllner et al., 1993), and GAD65 (glutamic acid decarboxylase of 65 kD) (Christgau et al., 1992), the enzyme which synthesizes the inhibitory neurotransmitter GABA (Erlander and Tobin, 1991).

In mammals, glutamic acid decarboxylase (GAD)¹ is represented by two different isoforms defined as GAD67 and GAD65 according to their molecular masses of 67 and 65 kD, respectively (Kobayashi et al., 1987; Erlander et al., 1991). The two GAD isoforms are highly conserved in evolution and exhibit a substantial degree of homology (64% identity in the rat). Neither GAD65 nor GAD67 have putative transmembrane regions (Erlander and Tobin, 1991).

They differ considerably only in the NH₂-terminal portion (a.a. 1-100)—a region which does not appear to be essential for the catalytic activity of the molecule (Erlander et al., 1991). GAD65 and GAD67 are coexpressed in the large majority of GABA-secreting neurons (Erlander and Tobin, 1991), pancreatic islets (Vincent et al., 1983; Reetz et al.,

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1. *Abbreviations used in this paper:* a.a., amino acids; GAD, glutamic acid decarboxylase; GAD65, 65 kD isoform of glutamic acid decarboxylase; GAD67, 67 kD isoform of glutamic acid decarboxylase.

1991; Karlsen et al., 1991; Kim et al., 1993), and in few other cell types (Erdö et al., 1990; Persson et al., 1990; Tillakaratne et al., 1992). GAD65, is the dominant autoantigen in two human autoimmune diseases: Stiff-Man Syndrome, a rare neurological disease (Solimena et al., 1988, 1990; Butler et al., 1993; Björk et al., 1994); and insulin-dependent diabetes mellitus (Solimena et al., 1990; Baekkeskov et al., 1990; Hagopian et al., 1993; Richter et al., 1993; Velloso et al., 1993).

Although very similar, GAD65 and GAD67 have a partially different intracellular distribution. In neurons, GAD65 is enriched in nerve terminals (Kaufman et al., 1991) where a pool of the protein is associated with synaptic vesicles (Reetz et al., 1991). GAD67, on the other hand, has a more diffuse distribution throughout the neuronal cytosol (Kaufman et al., 1991). In pancreatic β -cells GAD65 is primarily concentrated in the region of the Golgi complex (Reetz et al., 1991; Sorenson et al., 1991) and in close proximity to synaptic-like microvesicles (Reetz et al., 1991; Christgau et al., 1992). Subcellular fractionation studies carried out in pancreatic islets have demonstrated that GAD65 is partially recovered in particulate fractions, while GAD67 is recovered in soluble fractions (Christgau et al., 1991). The differential targeting of the two GAD isoforms has also been observed in fibroblasts independently transfected with GAD65 and GAD67. In these cells GAD65 was concentrated in the Golgi complex region and was partially recovered in particulate fractions in subcellular fractionation experiments, while GAD67 was found homogeneously distributed throughout the cell cytosol and was recovered only in soluble fractions (Solimena et al., 1993).

The limited primary sequence divergence between GAD65 and GAD67 facilitates the identification of signal(s) involved in the targeting of GAD65 to subcellular structures. Studies carried out in pancreatic islets and insect Sf9 cells transfected with GAD65 have shown that GAD65 undergoes two hydrophobic posttranslational modifications within its first 100 a.a. One of the two modifications was identified as palmitoylation sensitive to hydroxylamine treatment at pH 8 (Christgau et al., 1992), a result suggestive of a thio-ester linkage (James and Olson, 1990; Masterson and Magee, 1991). Proteolytic removal of this NH₂-terminal region of GAD65 led to a complete solubilization of the remaining fragment, suggesting that the recovery of GAD65 in particulate fractions was due to an association of the protein with membranes mediated by a lipid anchor (Christgau et al., 1992). When CHO cells were transfected with a construct in which the NH₂-terminal region of rat GAD67 (a.a. 1-88) was replaced by the corresponding portion of rat GAD65 (a.a. 1-83), the chimeric GAD molecule, like wild-type GAD65, was concentrated in the region of the Golgi complex (Solimena et al., 1993). This finding suggests that the NH₂-terminal domain of GAD65 contains information sufficient to target the otherwise soluble GAD67 to this intracellular compartment.

In this study we have further defined the mechanisms responsible for the intracellular targeting of GAD65. We demonstrate that its NH₂-terminal domain (a.a. 1-83) is sufficient to target an unrelated protein, β -galactosidase, to the Golgi complex region. Using site-directed mutagenesis, we show that thioacylation is not required for the targeting of the protein to this cellular region. In addition, we demonstrate

that a domain corresponding to a.a. 1-27 of GAD65, which does not include any cysteine, is required to target the otherwise soluble GAD67 to the Golgi complex region. Conversely, a chimeric protein in which a.a. 1-27 of GAD65 were replaced by the corresponding region of GAD67 (a.a. 1-29) displayed a homogeneously diffuse distribution in the cell cytosol. We suggest that a.a. 1-27 contain a signal which is necessary for targeting GAD65 to the Golgi complex region.

Materials and Methods

Antibodies and Cytochemical Probes

Rabbit antiserum 7673 raised against the last 17 a.a. of rat GAD67 and which recognizes both GAD67 and GAD65 was previously described (Solimena et al., 1993). The following antibodies were generous gifts: mouse monoclonal antibody GAD6 directed against GAD65 (Dr. D. Gottlieb, St. Louis, MO; Chang and Gottlieb, 1988); rabbit serum directed against rat synaptophysin (Dr. R. Jahn, New Haven, CT) (Navone et al., 1986); rabbit serum directed against α -mannosidase II (Dr. Moremen, Athens, GA) (Moremen and Touster, 1985); rabbit serum directed against TGN38 (Dr. Howell, Denver, CO) (Jones et al., 1993). The following reagents were obtained from commercial sources: K2 rabbit antiserum raised against recombinant GAD67 (Chemicon, Temecula, CA); mouse monoclonal antibodies directed against β -galactosidase (Promega, Madison, WI); FITC-conjugated lentil lectin (*Lens culinaris*) (EY Laboratories, San Mateo, CA); secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA).

Generation of GAD Constructs

Standard DNA procedures were performed according to Sambrook et al. (1989). Cloning of rat GAD65 cDNA was previously described (Solimena et al., 1993). The rat GAD67 cDNA was a generous gift of Dr. D. Gottlieb (St. Louis, MO) (Wyborski et al., 1990). GAD65 and GAD67 cDNA were excised from Bluescript II SK +/- (Stratagene, La Jolla, CA) as HindIII-NotI (GAD65) and HindIII-HindIII (GAD67) fragments and subcloned into the pRC/RSV vector (Invitrogen, San Diego, CA) for expression in CHO cells. Generation of the fusion protein GAD65(1-83)/GAD67 was previously described (Solimena et al., 1993). Site-directed mutagenesis to replace cysteines with serines at a.a. positions 30, 45, 73, 75, 80, and 82 of GAD65 (see Fig. 1) was performed according to PCR procedures previously described (Higuchi, 1989). The resulting constructs are referred to as GAD65 S1, GAD65 S2, etc. (Fig. 1). The same approach was used to generate GAD65 S1-6, in which all the cysteines contained within a.a. 1-82 of GAD65 were replaced by serines. Similar PCR procedures, in this case using overlapping GAD65/GAD67, GAD67/GAD65, and GAD/ β -galactosidase fusion primers, were used to generate the following chimeric GAD constructs: GAD65(1-27)/GAD67, in which a.a. 1-27 of GAD65 replaced the corresponding region (a.a. 1-29, see Fig. 1) of GAD67; GAD65(1-57)/GAD67, in which a.a. 1-57 of GAD65 replaced the corresponding region (a.a. 1-61) of GAD67; GAD67(1-29)/GAD65, in which a.a. 1-29 of GAD67 replaced the corresponding region (a.a. 1-27) of GAD65; GAD65(1-83)/ β -galactosidase, in which a.a. 1-83 of GAD65 were fused to the NH₂-terminus of β -galactosidase. The β -galactosidase cDNA was a kind gift of Dr. M. Lerner (Yale University, New Haven, CT). The sequence of all PCR products was confirmed by sequence analysis, and the resulting constructs were subcloned into the pRC/RSV vector for transfection.

Hippocampal Cell Cultures

Primary culture of hippocampal neurons were prepared as previously described (Banker and Cowan, 1977; Goslin et al., 1990; Mundigl et al., 1993). Cells were plated at the density of 4,000 \times cm² on coverslips and grown up to five weeks.

Stable Transfection of CHO Cells

Cultured CHO cells (Gluzman, 1981; Cameron et al., 1991) were transiently transfected using Lipofectin reagent (GIBCO/BRL, Gaithersburg, MD) as previously described (Solimena et al., 1993). Stable cell lines were cloned by limiting dilution. After two weeks in selective me-

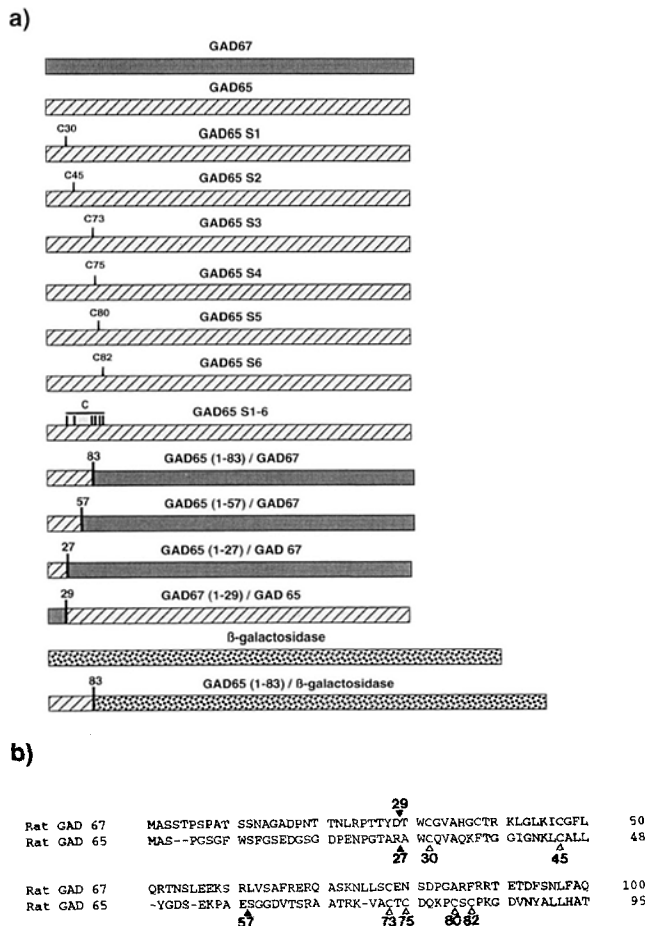


Figure 1. Schematic drawing illustrating the GAD constructs investigated in this paper. Cysteines which have been replaced by a serine are indicated by a C. (b) Sequence alignment of the NH₂-terminal region of GAD65 (a.a. 1-95) and GAD67 (a.a. 1-100). Open arrowheads point to each of the 6 cysteines contained within the NH₂-terminal region of GAD65. Closed arrowheads indicate the position at which GAD67 and GAD65 have been spliced to produce chimeric proteins.

dia (DMEM containing 700 μ g/ml of G418), transfected cells were treated for 18 h with 10 mM n-butyric acid and screened for level of expression of the transfected protein by Western blotting. At least three independent expressing clones of each construct were analyzed for localization of the protein by immunocytochemistry. To increase the levels of expression of the transfected protein, cells were incubated with 10 mM n-butyric acid for 24 h before fixation or homogenization.

Immunofluorescence

Single and double immunofluorescence staining was performed as previously described (Cameron et al., 1991; Solimena et al., 1993; Mundigl et al., 1993).

Biochemical Procedures

Subcellular fractionation. Stably transfected CHO cells were grown to confluency. Approximately 8×10^7 cells were harvested in 15 ml PBS, 5 mM EDTA, pelleted at 1,000 g for 10' at 4°C and resuspended in 3 ml of homogenization buffer (150 mM NaCl, 20 mM Hepes, pH 7.4 and the following protease inhibitor mixture: 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin in methanol, 1 μ g/ml antipain in ethanol, 1 mM PMSF in isopropanol, 10 mM benzamide). Cells were homogenized with 10 strokes in a ball bearing homogenizer, ball diameter 0.0016 inches, clear-

ance 0.0012 inches) (H. Issel Inc., Redwood, CA). Cell homogenates were centrifuged at 3,000 g for 10' at 4°C in a refrigerated microcentrifuge Sigma 2K15 (B. Braun, Allentown, PA). 2.5-ml aliquots of the resulting postnuclear supernatants were spun at 100,000 g for 20' at 4°C in a TL100.3 rotor (Beckman Instruments, Palo Alto, CA). The pellets were resuspended to the volume of the original postnuclear supernatant in homogenization buffer. High-speed supernatants and resuspended high-speed pellets were analyzed by SDS-PAGE and immunoblotted as described (Laemmli, 1970; Towbin et al., 1979).

Triton X-114 assay. The following protocol was modified from the original procedure described by Bordier (1981). Approximately 8×10^7 cells were washed once with PBS, and then solubilized in 2 ml solubilization buffer consisting of 150 mM NaCl, 1% precondensed Triton X-114, 20 mM Hepes, pH 7.4 and the following protease inhibitors mixture: 1 μ g/ml leupeptin; 1 μ g/ml aprotinin, 1 μ g/ml pepstatin in methanol, 1 μ g/ml antipain in ethanol, 1 mM PMSF in isopropanol, 10 mM benzamide. The detergent extracts were incubated for 1 h at 4°C on a nutator. The Triton X-114 insoluble material was pelleted by centrifugation at 100,000 g, 4°C for 30'. 0.5-ml aliquots of the Triton X-114 soluble material were overlaid in 1.5-ml microcentrifuge tubes onto 0.5 ml 6% sucrose cushions in solubilization buffer and incubated for 5' at 37°C. After centrifugation at 13,000 g for 10' at room temperature, the tubes were placed again at 37°C, the aqueous fractions removed (\sim 900 μ l each) and detergent phases brought back to 1 ml with 150 mM NaCl, 10 mM Hepes, pH 7.4. 100 μ l 10% Triton X-114 was added to each aqueous phase. Aqueous and detergent phases were analyzed by SDS-PAGE and immunoblotted. Immunoreactivity in Western blots was revealed either by enhanced chemiluminescence detection according to manufacturer's instruction (Amersham Corp., Arlington Heights, IL), or by ¹²⁵I-protein A (Du Pont New England Nuclear, Boston, MA) and autoradiography.

Scanning and densitometry of the autoradiograms was performed using the Bio Image program on a Visage 2000 scanner (Millipore Corp., Bedford, MA).

Results

A Pool of GAD65 Is Localized at the Golgi Complex Region in Neurons

In this study we have investigated the mechanisms responsible for the targeting of GAD65 to the region of the Golgi complex. This localization was previously demonstrated in pancreatic β -cells, which endogenously express the protein (Reetz et al., 1991), as well as in transfected CHO and COS cells (Solimena et al., 1993). To establish whether a pool of GAD65 is also concentrated at the Golgi complex region of neuronal cells, we studied the localization of the protein in cultured rat hippocampal neurons. In these cultures pyramidal neurons account for the majority of the cells, while GABA-ergic neurons, which express high level of GAD65, comprised \sim 7% of the neuronal population (Craig et al., 1993). GAD65 immunoreactivity became detectable after two weeks in culture and progressively increased both in the cell soma and in the axonal processes (data not shown). In all GABA-ergic neurons after 5 wk in culture, a pool of GAD65 was colocalized with TGN38, a marker protein of the Golgi complex (Luzio et al., 1990) (Fig. 2 b). Prominent GAD65 immunoreactivity was also visible in the presynaptic axonal varicosities of the same neurons, where synaptic vesicles are clustered.

The NH₂-terminal Region of GAD65 Is Sufficient to Target a GAD-unrelated Protein to the Region of the Golgi Complex

We have previously shown that the replacement of a.a. 1-88 of GAD67 with a.a. 1-83 of GAD65 is sufficient to target the otherwise soluble GAD67 to the region of the Golgi complex (Solimena et al., 1993). This experiment demonstrated that

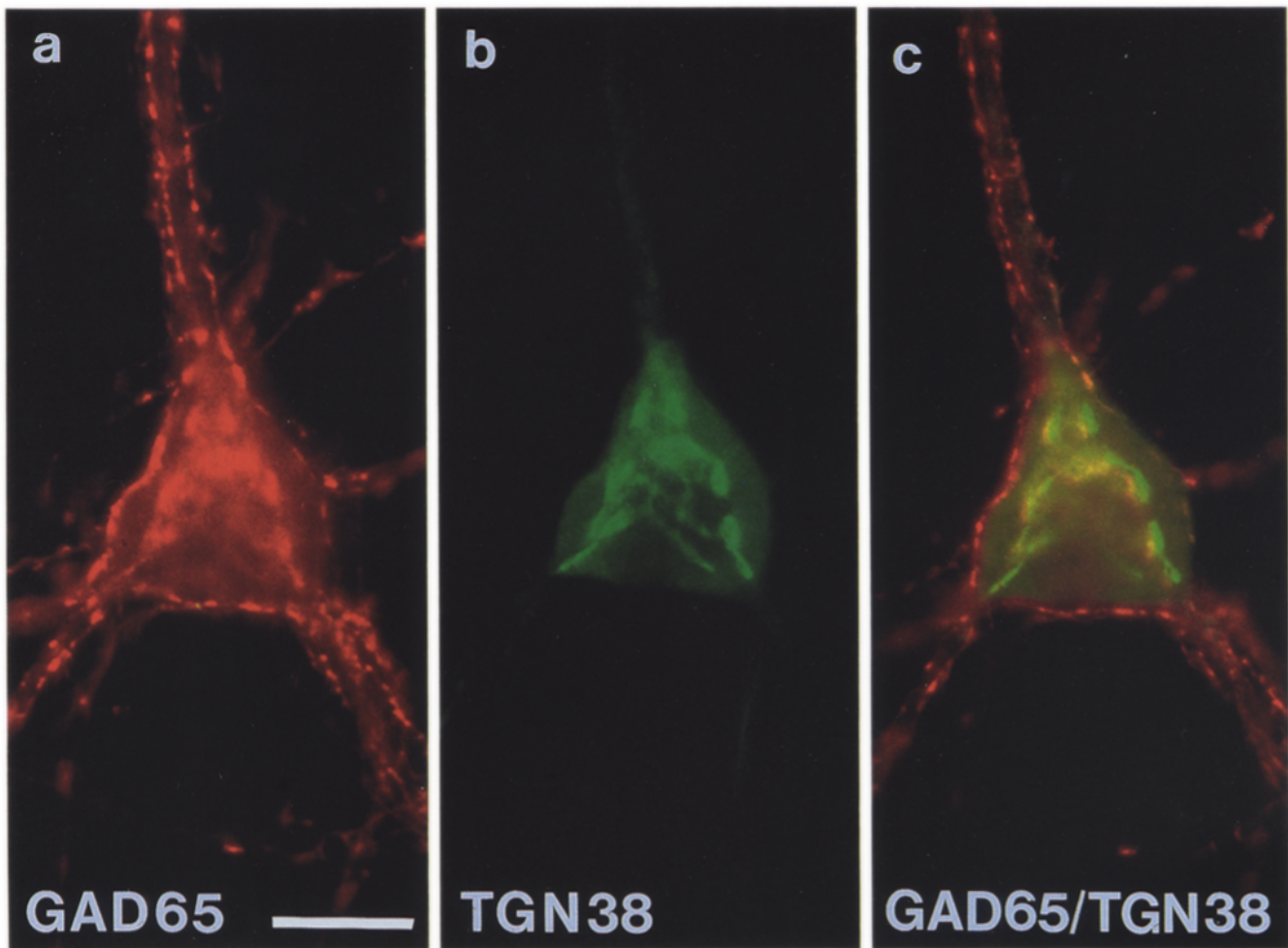


Figure 2. Color micrograph showing the localization of GAD65 in the Golgi complex of a hippocampal neuron in primary culture. Neurons from a 5-wk old culture were double-labeled for GAD65 with the GAD65-specific monoclonal antibody GAD6 (a, red fluorescence) and for the Golgi marker TGN38 (b, green fluorescence). GAD65 and TGN 38 immunoreactivities precisely coincide in the region of the Golgi complex. c shows a superimposition of the two staining patterns. GAD65 and TGN 38 immunoreactivities precisely coincide in the region of the Golgi complex (yellow fluorescence in c). In addition, GAD65 is also concentrated in presynaptic varicosities of axons which innervate the perikarya dendrites of the neuron. Bar, 17 μ m.

the NH₂-terminal region of GAD65 is required for its intracellular sorting. However, it did not rule out the possibility that some features conserved between the two GAD isoforms may act in concert with the NH₂-terminal domain of GAD65 to determine its subcellular targeting. To rule out this possibility, we tested whether a.a. 1-83 are sufficient to target an unrelated protein, β -galactosidase, to the same intracellular compartment as GAD65. CHO cells were transiently transfected either with β -galactosidase or with the GAD65(1-83)/ β -galactosidase fusion protein, in which a.a. 1-83 of GAD-65 were linked to the NH₂ terminus of β -galactosidase. While β -galactosidase had a diffuse cytosolic localization (Fig. 3 a), GAD65(1-83)/ β -galactosidase was concentrated in the region of the Golgi complex (Fig. 3, b and c), as demonstrated by its colocalization with a Golgi marker (lentil lectin-binding sites, Ridgway et al., 1992) (compare Fig. 3 c with Fig. 3 d).

Targeting of GAD65 to the Golgi Complex Region Does Not Depend on Thio-Palmitoylation

Palmitic acid bound to the NH₂-terminal region of GAD65

can be removed by treatment with hydroxylamine at pH 8.0 (Christgau et al., 1992). This finding suggests a binding of the fatty acid mediated by a thio-ester linkage (James and Olson, 1990; Masterson and Magee, 1991). Having established that a.a. 1-83 of GAD65 contains the information which is necessary and sufficient to target a protein to the Golgi complex region, we investigated whether preventing thio-acylation within this domain would affect the protein's intracellular distribution. This region of GAD65 contains 6 cysteines at positions 30, 45, 73, 75, 80, and 82 (Fig. 1 b). To test the role of thio-acylation in GAD65 targeting, we generated CHO cell lines that stably express GAD65 constructs in which each of the 6 cysteines was replaced by serine residues (Fig. 1). All the constructs harboring a single point mutation were still targeted to Golgi complex region, as demonstrated by their colocalization with Golgi markers including lentil lectin-binding sites (not shown) and α -mannosidase II (Moremen and Touster, 1985) (Fig. 4). Since palmitoylation may in principle occur on more than one cysteine simultaneously, we also generated cell lines permanently expressing GAD65 S1-6, a GAD65 mutant in which all of the

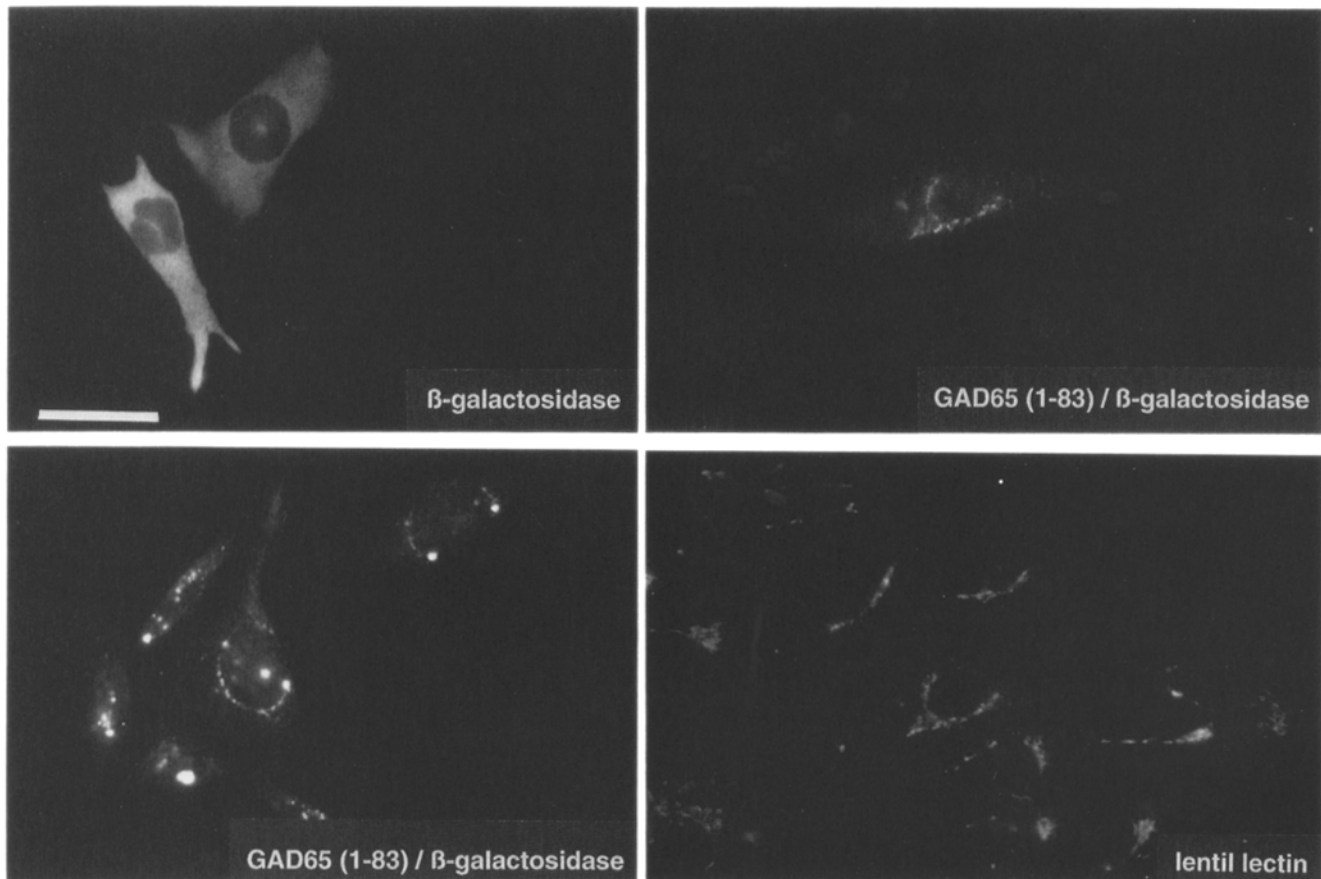
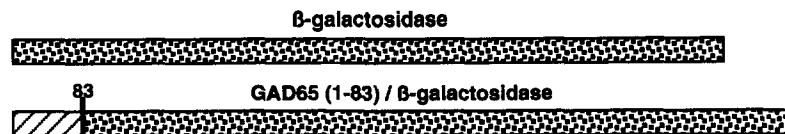


Figure 3. Intracellular distribution of β -galactosidase and of GAD65(1-83)/ β -galactosidase in transiently transfected CHO cells. Cells transfected with β -galactosidase. Cells transfected with GAD65(1-83)/ β -galactosidase. β -galactosidase has a diffuse cytosolic distribution while GAD65(1-83)/ β -galactosidase is concentrated in the region of the Golgi complex. Shows counterstaining of the field shown in GAD65(1-83)/ β -galactosidase with FITC-labeled lentil lectin, a lectin which specifically binds to the Golgi complex region. Only one of the cells present in the field expresses GAD65(1-83)/ β -galactosidase. Bar, 25 μ m.

6 cysteines were replaced by serines (Fig. 1). A significant pool of the protein was still targeted to the region of the Golgi complex (Fig. 5). These findings suggest that thio-palmitoylation of GAD65 is not required for the targeting of GAD65 to the Golgi complex region.

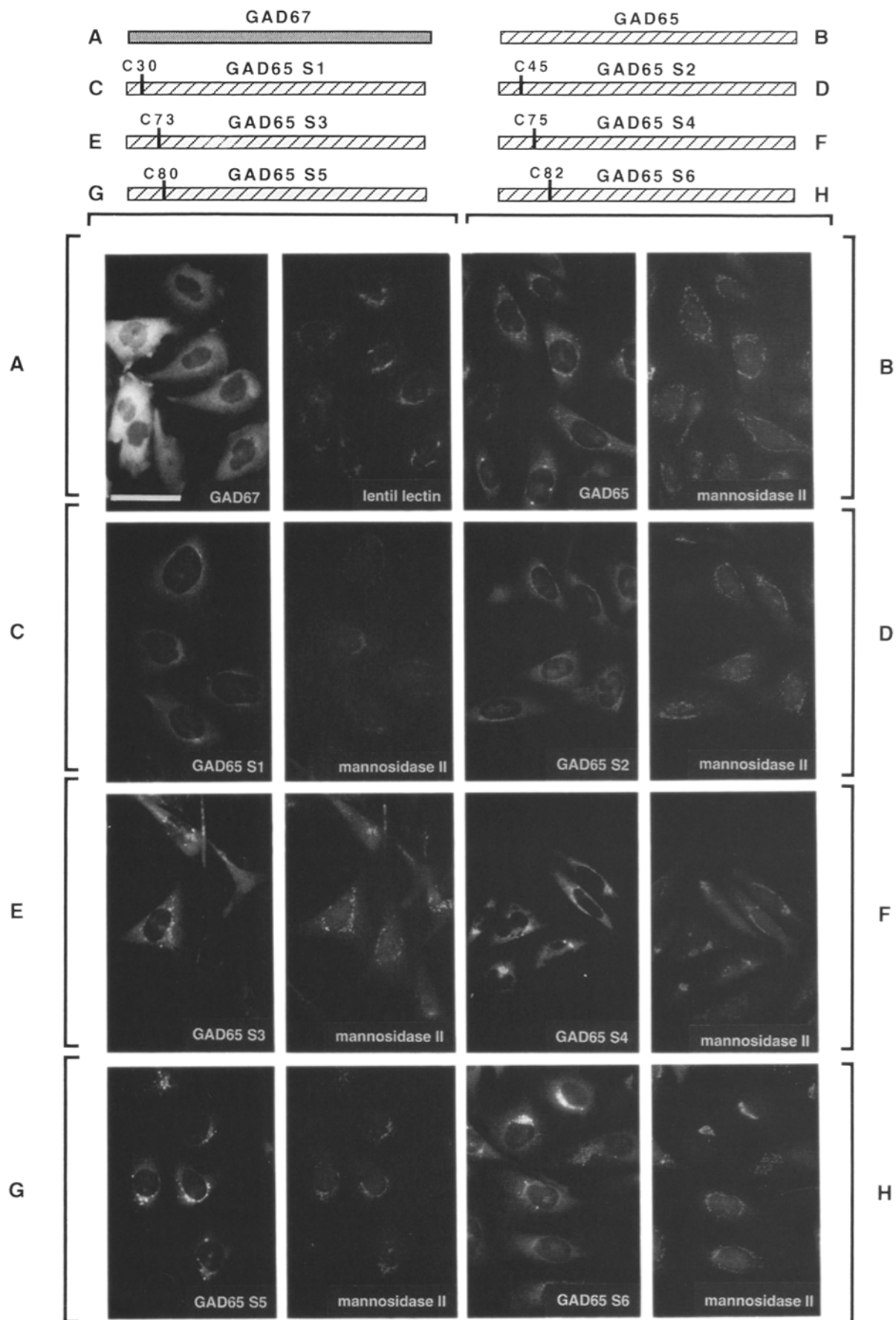
Amino Acids 1-27 of GAD65 Contain a Signal Required for the Targeting of GAD65 to the Golgi Complex Region

We next tried to further define the domain at the NH₂-terminal region of GAD65 responsible for its targeting to the Golgi complex region. Chimeric GAD proteins in which a.a. 1-57 or 1-27 of GAD65 replaced the corresponding regions in GAD67, i.e., constructs GAD65(1-57)/GAD67 and GAD65(1-27)/GAD67, respectively (Fig. 1 and Fig. 6, *a* and *c*), were still colocalized with Golgi complex markers in the perinuclear region when expressed by stable transfection in CHO cells (Fig. 6, *b* and *d*). Conversely, replacement of a.a. 1-27 of GAD65 with the corresponding region of GAD67

resulted in a chimeric protein referred to as GAD67(1-29)/GAD65 (Fig. 1 and Fig. 6 *e*), which has a diffuse cytoplasmic localization resembling that of wild-type GAD67. These results indicate that a.a. 1-27 of GAD65 encode a signal which is required for the targeting of the protein to the area of the Golgi complex.

Recovery in Particulate Fractions and Hydrophobicity of GAD65 Rely on a.a. 1-27

To complement our immunofluorescence data, the distribution between soluble and particulate fractions of cell homogenates for each of the constructs described above was analyzed in stably transfected CHO cell lines. As previously reported (Solimena et al., 1993), virtually all GAD67 was recovered in the soluble fraction (high-speed supernatant) of the cell homogenate (Fig. 7, lanes 1 and 2). In contrast, ~40% of GAD65 was recovered in the particulate fraction (high-speed pellet) (Fig. 7, lanes 3 and 4). All constructs which comprised a.a. 1-27 of GAD65, i.e., GAD65 S1-6,



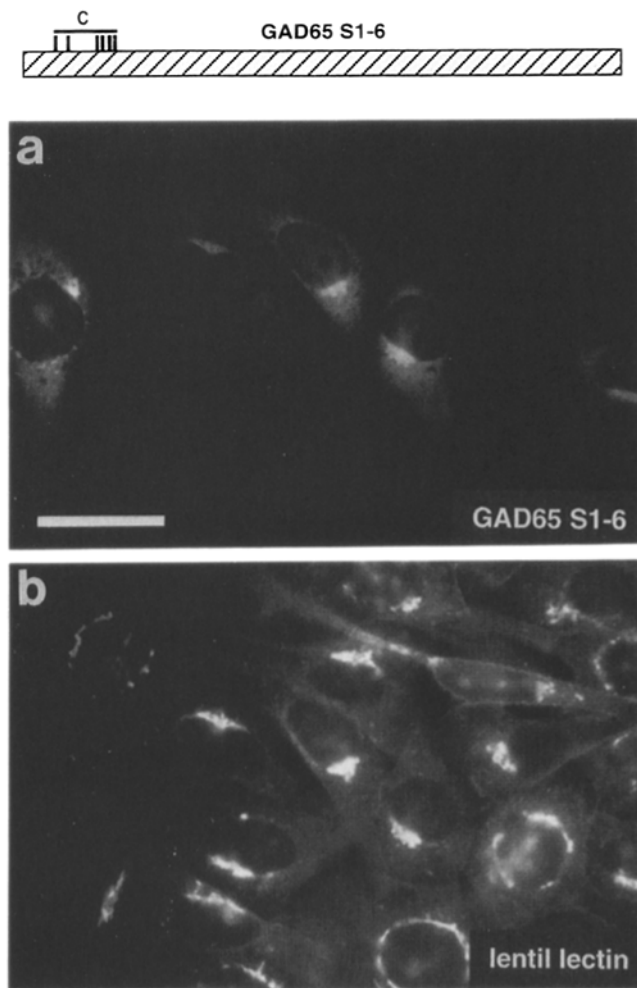


Figure 5. Targeting to the Golgi complex region of a GAD65 mutant protein in which all cysteines located within a.a. 1-100 have been replaced by serines (GAD65 S1-6). Stably transfected cell line in which GAD65 S1-6 is expressed by a subpopulation of cells. Cells were stained with the monoclonal antibody GAD6 (*a*) and counterstained with FITC-lentil lectin (*b*). Bars, 28 μ m.

GAD65(1-83)/GAD67 and GAD65(1-27)/GAD67 were partially recovered in pellet fractions although to a lower extent than GAD65 (Fig. 7, lanes 5-10). Conversely, virtually all GAD67(1-29)/GAD65 was recovered in the soluble fraction (Fig. 7, lanes 11 and 12). These results, which are in agreement with immunofluorescence data, emphasize the crucial role of a.a. 1-27 of GAD65 in determining its particulate distribution, although additional structural elements beyond a.a. 1-27, including thio-acylation, may further contribute to membrane association of GAD65.

The hydrophobicity of the GAD constructs was analyzed by the Triton X-114 detergent assay (Fig. 8). In agreement

with previous studies (Christgau et al., 1991, 1992; Solimena et al., 1993), GAD67 only partitioned in the aqueous phase (Fig. 8, lanes 1 and 2), while GAD65 partitioned in roughly equal amounts between the aqueous and the detergent phase (Fig. 8, lanes 3 and 4). The chimera GAD65(1-83)/GAD67 was recovered in both phases (Fig. 8, lanes 7 and 8), although the ratio between detergent and aqueous pools of the protein was lower than in the case of GAD65. This construct does not contain the region corresponding to a.a. 325-355 of GAD65 which appear to be considerably more hydrophobic than the corresponding region of GAD67 (Karlsen et al., 1991). Palmitoylation of the NH₂-terminal GAD65 fragment of this construct may therefore explain its partial hydrophobicity. On the other hand, a similar detergent/aqueous ratio was observed for GAD65(1-27)/GAD67 (Fig. 7, lanes 9 and 10). This finding suggests either that a.a. 1-27 may undergo hydrophobic modification in spite of the absence of cysteines in this region, or that this a.a. stretch confers upon the adjacent portion of GAD67 the property to become a suitable substrate for thio-acylation. The former possibility is supported by the similar recovery in the detergent phase of the GAD65 S1-6 and GAD65 (1-27)/GAD67. Taken together these findings support the hypothesis that some other hydrophobic modification(s), distinct from thioacylation contribute to the hydrophobicity of GAD65 (Christgau et al., 1992). Finally, virtually all GAD67(1-29)/GAD65 protein, which has a soluble cytosolic intracellular distribution (Fig. 6 *e* and Fig. 7, lanes 11 and 12), was recovered in the aqueous phase (Fig. 8, lanes 11 and 12).

Discussion

In this study we have investigated the molecular mechanisms responsible for the differential targeting of two very similar proteins, GAD65 and GAD67, within the cell cytosol. The removal of all the potential cysteine acceptor site(s) for thio-acylation within the domain of GAD65 known to undergo palmitoylation did not affect targeting of the protein to the Golgi complex region. Conversely, the replacement of a.a. 1-27 of GAD65 with the corresponding region of GAD67 abolished this localization. Our results indicate that the NH₂-terminal 27 a.a. of GAD65 are required for its targeting to the region of the Golgi complex. With all constructs investigated, results of immunofluorescence were always consistent with results of subcellular fractionation. All GAD proteins which were targeted to the Golgi complex region were also partially recovered in pellets after high-speed centrifugation of cell homogenates, and were at least partially hydrophobic.

Secondary structure predictions using a variety of algorithms (Chou and Fasman, 1978; Rose, 1978; Garnier et al., 1978) did not reveal structural motifs in a.a. 1-27 of GAD65 (for example possibility of an amphiphatic α -helix) which may mediate membrane interaction. This region in-

Figure 4. Gallery of micrographs demonstrating the intracellular localization of wild-type and mutant GAD proteins in transfected CHO cell lines stably expressing each of the protein. Each cell line was stained by GAD (TRITC fluorescence) and counterstained with either lentil lectin or anti- α -mannosidase antibodies (FITC fluorescence) to reveal the morphology of the Golgi complex. GAD65 was stained with the GAD65-specific monoclonal antibody GAD6. GAD67 was stained with the rabbit K2 antibody. (*A*) GAD67 has a diffuse distribution in the cell cytosol. (*B*) GAD65 is concentrated in the region of the Golgi complex. (*C-H*) GAD65 mutant proteins with cysteine \rightarrow serine point-mutations at each of the cysteine localized within a.a. 1-100 of the molecule (GAD65 S1-GAD65 S6). Bars, 36 μ m.

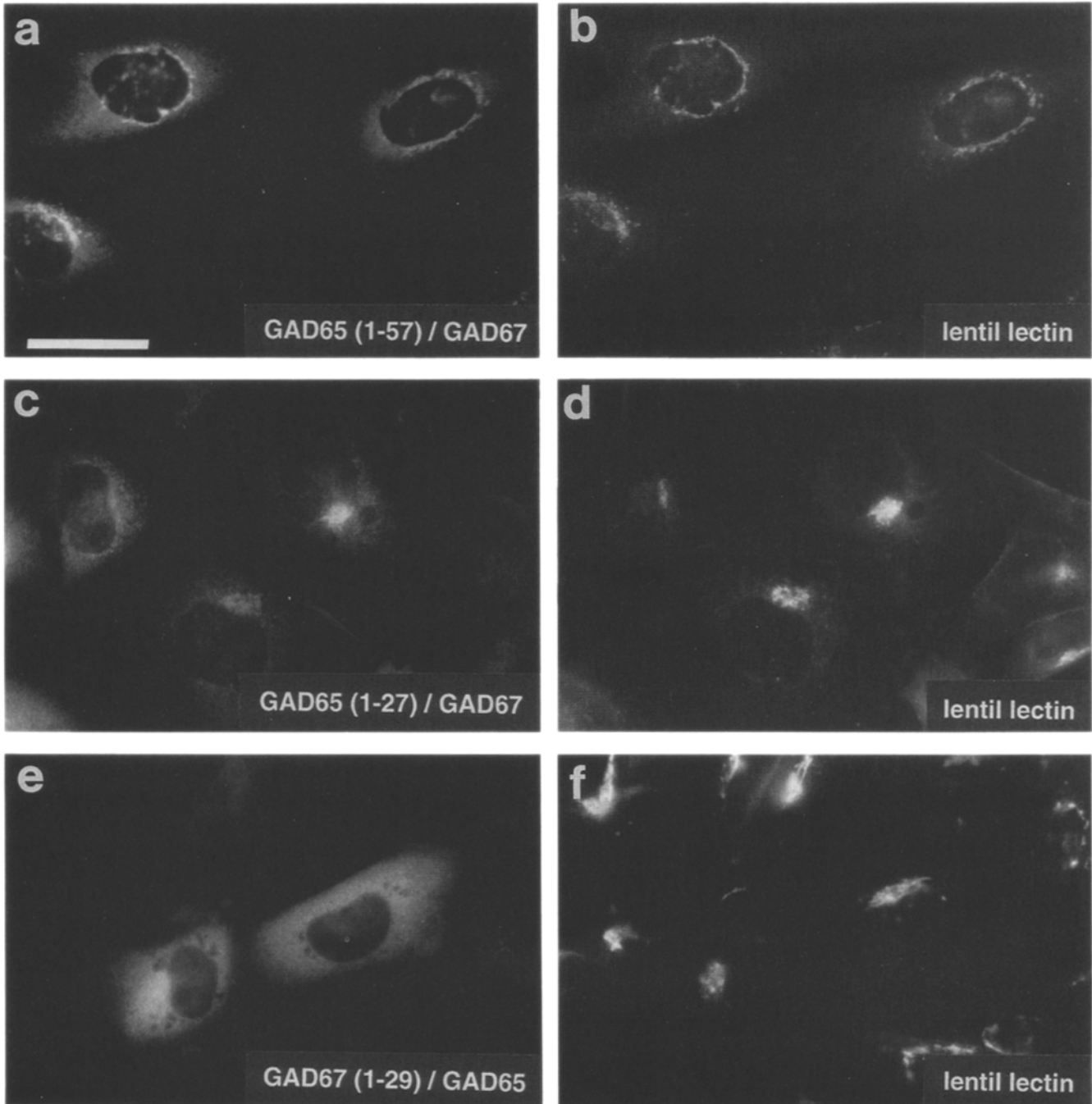
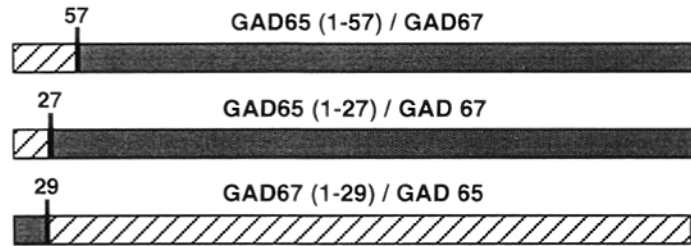


Figure 6. Intracellular distribution in stably transfected cells of GAD65/GAD67 chimeric proteins. Cells were stained for GAD (antibody K2 for fields *a* and *c*; monoclonal antibody GAD6 for field *e*) and counterstained with FITC-lentil lectin. (*a* and *b*) Cells expressing GAD65(1-57)/GAD67. (*c* and *d*) Cells expressing GAD65(1-27)/GAD67. (*e* and *f*) Cells expressing GAD67(1-29)/GAD65. GAD proteins containing the NH₂-terminal region of GAD65 are colocalized with lentil lectin in the Golgi complex region. The different morphology of the Golgi complex in *a* and *b*, and in *c* and *d*, reflects a normal variation of the architecture of the Golgi complex in CHO cells. The GAD fusion protein containing the NH₂-terminal region of GAD67 has a diffuse distribution in the cytosol. Bars, 22 μ m.

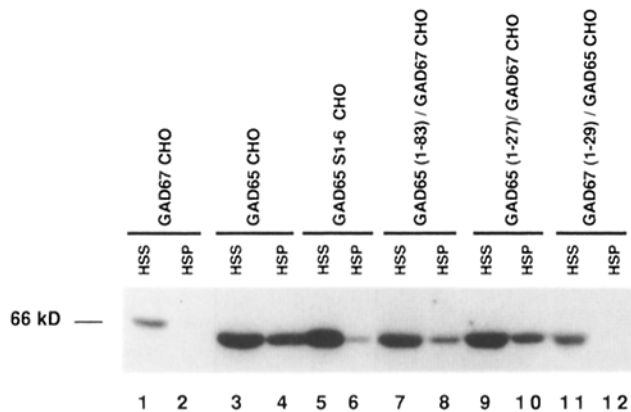


Figure 7. Distribution of GAD67, GAD65, and GAD mutant proteins in particulate and soluble fractions of stably transfected CHO cell homogenates. *HSS*, high speed supernatants; *HSP*, high speed pellets. The percentage of each construct in the high speed supernatant and in the high speed pellets, as determined by densitometry, was the following: *GAD67*, 99/1; *GAD65*, 63/37; *GAD65 S1-6*, 91/9; *GAD65 (1-83)/GAD67*, 82/18; *GAD (1-27)/GAD67*, 76/24; *GAD67 (1-29)/GAD65*, 97/3.

cludes three prolines. There are at least two possible mechanisms through which a.a. 1-27 of GAD65 may target the protein to the Golgi complex region. This a.a. stretch may target GAD65 via an interaction with a binding protein(s). Since targeting of GAD65 to the region of the Golgi complex occurs not only in neurons and in pancreatic β -cells, but also in cells that do not express the protein constitutively such as fibroblasts, an implication of this hypothesis is that the putative GAD65-binding protein(s) should be present in all cells.

Alternatively, a hydrophobic posttranslational modification within this region other than thio-palmitoylation could be involved. It has been proposed that the hydrophobicity of GAD65 is the consequence of two distinct posttranslational modifications occurring within a.a. 1-100 of the protein: palmitoylation sensitive to hydroxylamine treatment at pH 8 and another yet to be characterized hydrophobic modification (Christgau et al., 1992). Sensitivity to hydroxylamine at pH 8 strongly suggests that palmitic acid is linked by a thio-ester bond (i.e., to cysteine residues) (James and Olson, 1990; Masterson and Magee, 1991). Amino acids 1-100 of GAD65 contains 6 cysteines at positions 30, 45, 73, 75, 80, and 82. Our results are consistent with the occurrence of another posttranslational modification in addition to thio-acylation, since replacement of all the 6 cysteines decreased, but did not abolish, hydrophobicity of the resulting protein.

An intriguing observation is that virtually no GAD67(1-29)/GAD65, which still includes the 6 cysteines, was recovered in the detergent phase. This finding may have two explanations. One possibility to explain this finding is that a.a. 1-27 of GAD67 are required for an efficient recognition of GAD65 as a substrate for palmitoylation. Another explanation, suggested by the diffuse intracellular localization of this chimeric protein and by its recovery in the soluble cytosolic fraction, is that a.a. 1-27 are necessary to target the protein to sites where the palmitoyltransferase is localized. The presence of palmitoyltransferase activity in the region of the Golgi complex has been reported (Schmidt and Catterall, 1987). Interestingly, also in the case of proteins of the RAS

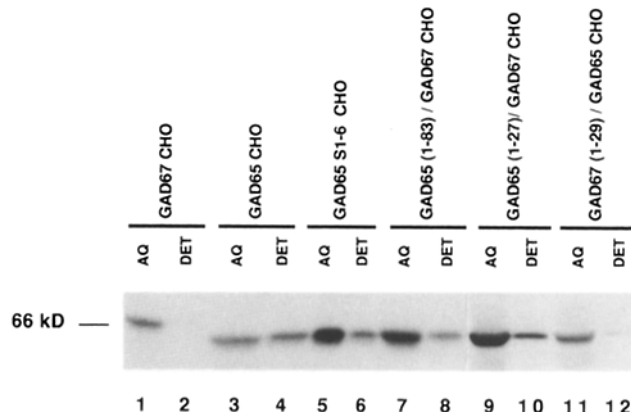


Figure 8. Partitioning of GAD67, GAD65, and GAD mutant proteins in the aqueous (AQ) and detergent phase (DET) after Triton X-114 extraction and phase separation. Percentage of each construct in the aqueous and in the detergent phase, as determined by densitometry, were the following: *GAD67*, 99/1; *GAD65*, 53/46; *GAD65 S1-6*, 79/21; *GAD65 (1-83)/GAD67*, 82/18; *GAD (1-27)/GAD67*, 79/20; *GAD67 (1-29)/GAD65*, 99/1.

superfamily, palmitoylation occurs only if the protein contains another membrane-targeting signal (Hancock et al., 1989).

While our data speak against a primary role of palmitoylation of cysteine(s) residues in the targeting of GAD65 to the region of the Golgi complex, and in general to particulate fractions, they do not rule out a role of palmitoylation in strengthening the interaction of GAD65 with subcellular particles, most likely membranes. Among the constructs containing a.a. 1-27 of GAD65, the less effective recovery in the high speed pellet was indeed observed in the case of GAD65 S1-6, in which all the potential acceptor sites for thioacylation had been replaced by serines.

It is of interest in this context to consider the multiple localizations of GAD65 in neurons and in β -cells. In neurons GAD65, besides being present in the area of the Golgi complex, is concentrated in nerve terminals where a pool of the protein is colocalized with synaptic vesicles (Reetz et al., 1991). In pancreatic β -cells a fraction of GAD65 is present in close proximity to GABA-containing synaptic-like microvesicles (Reetz et al., 1991). In principle these other localizations may be mediated by the same signal that targets GAD65 to the Golgi complex region. Another possibility is that GAD65, as a result of its accumulation in the region of the Golgi complex, is subsequently delivered to peripheral destinations via vesicles originating in this region. Palmitoylation could be relevant for GAD binding to these vesicles. On the other hand, immunocytochemistry of cultured neurons and of frozen brain sections demonstrate that the localization of GAD65 in nerve terminals include, but is not limited to, the sites where synaptic vesicles are clustered (our unpublished observations). Thus, an interaction of GAD with the cytomatrix surrounding these vesicles, rather than, or in addition to, an association with synaptic vesicles is possible. Notably, virtually no GAD is recovered on purified synaptic vesicles (Reetz et al., 1991) in spite of the observation that a considerable portion of brain GAD65 sediments upon high speed centrifugation (Chang and Gott-

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leishmania tubulin:      20          30          [43%]
                        GSKFWEVIADEHGVDPTGS
                        |||::: |||::: |||:::
rat GAD65 (aa 1-27): MASPGSGFWSFGSEDGSGDPENPGTAR
                        |::: |||::: |||:::
human tubulin beta-5 chain: GAKFWEVISDEHGIDP          [40%]
                        20          30

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Figure 9. Sequence similarity between a.a. 1-27 of rat GAD65 (*center*), a.a. 17-35 of leishmania tubulin (*top*), and a.a. 17-32 of human tubulin beta-5 chain (*bottom*). Search in the Swissprotein Database for sequences homologies to a.a. 1-27 of GAD65 was performed using the program FASTA (Wilbur and Lipman, 1983). Bars connected identical residues; dots connected homologous residues. Identity scores are indicated on the right. Note that the FASTA program considers residues at positions 14 (*E*, GAD65) and 26 (*D*, tubulins) and at positions 15 (*D*, GAD65) and 27 (*E*, tubulins) as identical.

lieb, 1988) and is hydrophobic (Reetz, A., and P. De Camilli, unpublished observations), consistent with an indirect interaction of GAD65 with synaptic vesicle membranes.

The property of GAD65 to serve as a substrate for palmitoylation is shared by other cytosolic neuronal proteins which are concentrated in axons and axon terminals: GAP-43 and SNAP-25. These two proteins as well are concentrated in the region of the Golgi complex (Goslin et al., 1990; Van Lookeren Campagne et al., 1992; M. C. Wilson, personal communications, The Scripps Research Institute, San Diego, CA; our unpublished observations). As in the case of GAD65, the sorting signal of GAP-43 is localized at its NH₂-terminal region which includes the two cysteine residues that undergo palmitoylation (Skene and Virag, 1989; Liu et al., 1991). However, no homology between the NH₂-terminal of GAD65 and GAP-43 has been found. In the case of SNAP-25, palmitoylation occurs in the region of the so-called cysteine quartet localized between a.a. 84-92 (Hess et al., 1992) within a short domain that is subjected to alternative splicing (Bark, 1993). The possibility should be considered that also in the case of these other proteins, palmitoylation may not be the primary targeting signal. It was shown that thio-palmitoylation of several neuronal and nerve terminal proteins including GAP-43 and SNAP-25 is reversible in nerve terminals and that the state of palmitoylation of a protein can be regulated (Hess et al., 1993). A spontaneous de-acylation of GAD65 was reported in β -cell extracts (Christgau et al., 1992).

Search in databases for sequence similarities revealed that a.a. 1-27 of GAD65 is similar to a domain in the NH₂-terminal region of β -tubulin (Fig. 9) which is contained within one of the most highly conserved β -tubulin regions (a.a. 1-60) (Cleveland and Sullivan, 1985). In animal cells microtubules originate (minus end) from the centrosomal area, which acts as the microtubule-organizing center (Mitchison and Kirschner, 1984; Bornens et al., 1987) and as the organizer of the structure of the Golgi complex (Tassin et al., 1985). It is of interest that in the ciliated epithelial cells of the fallopian tubes, another cell type which endogenously expresses GAD65 (Erdö et al., 1990; Tillakaratne et al., 1992), a pool of the protein is concentrated at basal bodies (Erdö et al., 1990; our unpublished observations) which are the organizers for the microtubular cytoskeleton of the cilia. It is possible that the similarity between a domain of β -tubulin and a domain of GAD65 may be related to the localization of GAD65 in proximity of microtubule-organizing centers.

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