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Association of GAD-65, but not of GAD-67, with the Golgi complex of transfected Chinese hamster ovary cells mediated by the N-terminal region

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ABSTRACT **Glutamic acid decarboxylase (GAD) is the enzyme responsible for synthesis of the neurotransmitter γ-aminobutyric acid in neurons and pancreatic β cells. It is represented by two isoforms, GAD-65 and GAD-67, which are the products of two different genes and differ substantially only at their N-terminal regions. GAD-65 is a dominant autoantigen in stiff-man syndrome and insulin-dependent diabetes mellitus. In neurons and β cells, GAD is concentrated around synaptic vesicles and synaptic-like microvesicles, respectively, as well as in the area of the Golgi complex. The mechanisms responsible for specific targeting of GAD to these organelles are not yet understood. The elucidation of the mechanism of subcellular targeting of GAD may be relevant to understanding its role as an autoantigen. In this study, the cloned genes for GAD-65 and GAD-67 were expressed separately in Chinese hamster ovary (CHO) cells and COS cells. While GAD-67 had a diffuse cytoplasmic localization, GAD-65 had a punctate distribution, with most of the immunoreactivity being concentrated in the area of the Golgi complex. A chimeric protein in which the 88 N-terminal amino acids of GAD-67 were replaced by the 83 N-terminal amino acids of GAD-65 was targeted to the Golgi complex, indicating that the N-terminal region of GAD-65 contains a targeting signal sufficient for directing the remaining portion of the molecule, highly similar in GAD-65 and GAD-67, to the Golgi complex-associated structures.**

Glutamic acid decarboxylase (GAD) is the enzyme responsible for synthesis of γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter of the brain (reviewed in ref. 1). GAD is expressed in neurons, in pancreatic β cells, and in cells of the testis and fallopian tube (2–5). In neurons and pancreatic β cells, GAD immunoreactivity is concentrated around synaptic vesicles and synaptic-like microvesicles, respectively, a localization that may facilitate the uptake of GABA into these vesicles (6). In addition, GAD immunoreactivity is detectable in the region of the Golgi complex (6, 7). The mechanism by which GAD is targeted to these organelles is not yet known.

There exist two major isoforms of GAD—GAD-65 and GAD-67 (65 and 67 kDa, respectively)—that are encoded by separate, unlinked genes (8, 9), both of which are expressed in neurons and β cells (6, 8–10). GAD-65 and GAD-67 differ substantially only in the first 100 amino acids at the N-terminal region (9). The primary amino acid sequences of the two isoforms show no conspicuous hydrophobic motif(s) that might account for membrane association. Studies on pancreatic islets have shown that GAD-65, but not GAD-67, is recovered in particulate fractions after high-speed centrifugation and partitions in the detergent phase after Triton X-114 phase separation (10). A pool of GAD-65 is palmitoylated within the first 100 amino acids at the N-terminal region (11). Immunostaining of nervous tissue with antibodies that recognize GAD-65 selectively, or GAD-67 preferentially, have suggested a differential distribution of the two isoforms in the neuronal cytoplasm and a greater concentration of GAD-65 in nerve terminals (12). Hence, the primary sequences of GAD-65 and GAD-67 may contain different targeting signals.

Elucidation of the subcellular distribution of the two GAD isoforms may have implications beyond the physiology of the GABA system. It has recently become clear that autoantibodies to GAD are frequently present in two autoimmune diseases, stiff-man syndrome and insulin-dependent diabetes mellitus (13–15). In stiff-man syndrome, these antibodies are primarily directed against GAD-65 (15, 16). The mechanisms by which proteins become autoantigens are unknown. In the case of GAD, a different localization of the two isoforms may contribute to their different autoantigenic properties.

To investigate the subcellular distribution of GAD and the mechanism underlying it more closely, the gene for GAD-65 was cloned, and its expression was compared with that of GAD-67 in transfected fibroblasts. We report that GAD-65, but not GAD-67, has an intrinsic capacity to associate with membranes, primarily with the Golgi complex. Transfection of a chimeric GAD-65–GAD-67 gene demonstrated that a signal sufficient for this association resides within the N-terminal domain of GAD-65.

MATERIALS AND METHODS

Antibodies. Synthetic peptides corresponding to amino acids 4–21 of rat GAD-65 and amino acids 577–593 of rat GAD-67 were used to raise rabbit antisera specific for GAD-65 (N65) or directed against both GAD-65 and GAD-67 (no. 7673). K2 rabbit antiserum, raised against recombinant GAD-67, was purchased from Chemicon. The following antibodies were generous gifts: rabbit antiserum against α-mannosidase II (K. Moremen, Cambridge, MA) (17); monoclonal antibodies against transferrin receptor (I. S. Trowbridge, San Diego) (18); monoclonal antibody that specifically recognizes GAD-65 (GAD-6) (D. Gottlieb, St. Louis) (19); rabbit antiserum raised against mouse recombinant GAD-67, which recognizes both GAD isoforms (no. 6799) (Z. Katarova and G. Szabo, Szeged, Hungary) (20).

Cloning of Rat GAD-65. The partial protein sequence of GAD-65 (formerly referred to as GAD-59) reported by Chang and Gottlieb (19) was used to design degenerate PCR primers [CCTCTAGAGCIAATACIAATACIAATGA(A/G)AT-OH and GGCTTAAGTAICGIGGIGGICCI(C/G)(A/T)(C/T)TT-OH], which facilitated amplification of a fragment of ≈150 bp from rat brain cDNA. By Northern blot analysis, the ^{32}P-labeled purified PCR fragment selectively hybridized to a band of ≈5.7 kb, present in brain but not liver RNA (Fig. 1).

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Abbreviation: GAD, glutamic acid decarboxylase.

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This result is consistent with the reported size of rat brain GAD-65 message (9). The same band was not recognized by a probe specific for rat GAD-67 (kindly provided by D. Gottlieb) (21). Sequence analysis (22, 23) of the PCR fragment was in agreement with the reported amino acid sequence of GAD-65 (19). Screening of 10^6 phage plaques from a Lambda ZAP II rat brain cDNA library (Stratagene) with the PCR fragment identified two independent, partially overlapping clones (0.95 and 2.2 kb, respectively), which together encoded 2.35 kb, including the full open reading frame of GAD-65. The sequence precisely matched that reported by Erlander *et al.* (9).

Subcloning of GAD-65, GAD-67, and GAD-65–GAD-67. Full-length clones of rat GAD-65 and GAD-67 (kind gift of D. Gottlieb) were subcloned into the pRC/RSV expression vector (Invitrogen, San Diego). A chimeric GAD-65–GAD-67 primer (AAAACCCTGCAGCTGCCCCCGCACAGAG-GAC-OH) and a GAD-67 specific primer (CAGAGAC-TCGGGGTGGTCAGACAGCTCCAA-OH) were used to generate by PCR a chimeric GAD-65–GAD-67 fragment of 225 bp using GAD-67 cDNA as a template. The *Pst* I/*Ava* I fragment of the chimeric PCR product was then inserted between a *Hin*dIII/*Pst* I fragment of rat GAD-65 and the *Ava* I/*Not* I fragment of rat GAD-67, resulting in replacement of amino acids 1–88 of GAD-67 with amino acids 1–83 of GAD-65 (Fig. 2). The resulting chimera was confirmed by sequence analysis and subcloned into the pRC/RSV vector.

Cell Cultures and Transfection. CHO cells (CHO-K1 cells) and COS-7 cells were grown as described (24, 25). For transient transfections, cells were seeded on coverslips and incubated on day 2 (≈40% confluency) with 5 μg of plasmid DNA and 15 μl of Lipofectin per ml (GIBCO/BRL) for 5–7 hr. On day 4, cells were fixed and processed for immunocytochemistry as described (24). To increase the levels of GAD expression, cells were treated from day 3 with 10 mM sodium butyrate. For stable transfection, cells were transfected on day 2 as described above and then incubated in the presence of 700 μg of G418 per ml (GIBCO/BRL) from day 4. CHO cell clones were obtained by limiting-dilution cloning and were screened for expression of GAD by Western blot analysis. Brefeldin A (Epicentre Technologies, Madison, WI) was added to culture medium in methanol at a final concentration of 5 μg/ml.

Biochemical Procedures. Triton X-114 extraction and phase separation were performed according to Bordier (26). Briefly, GAD-65 and GAD-67 CHO cell clones were grown to confluency, harvested, homogenized, and centrifuged as described (6). Postnuclear supernatants were spun at 100,000 × *g* for 20 min at 2°C in a TLA-100.2 rotor (Beckman). The pellets (high-speed pellets) were resuspended to the original postnuclear supernatant volume in homogenization buffer with fresh protease inhibitors. Both the resuspended pellets (high-speed pellets) and the supernatants (high-speed supernatants) were extracted with 2% (wt/vol) Triton X-114 on ice for 2 hr. Triton X-114-insoluble material was removed by centrifugation. Both Triton X-114-soluble extracts were processed for phase separation as described (26).

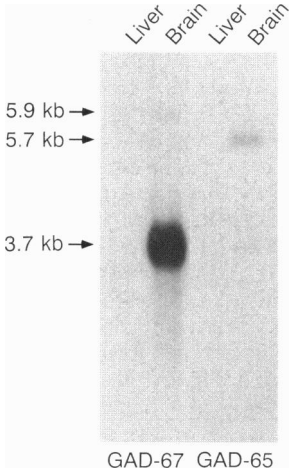

FIG. 1. Northern blot showing the hybridization of GAD-65 and GAD-67 probes with rat brain and liver poly(A)$^+$ RNA (10 μg per lane). The blot was first hybridized with the GAD-65 probe obtained by PCR, exposed for 72 hr with an intensifying screen at −80°C, stripped in boiling water, and then hybridized with the GAD-67 probe and reexposed for 72 hr. The GAD-65 probe obtained by PCR (*Right*) hybridizes with a brain message of 5.7 kb, but it does not recognize the major 3.7-kb and the minor 5.9-kb bands (20), which are hybridized by the GAD-67 probe (*Left*). Neither probe hybridizes with liver RNA.

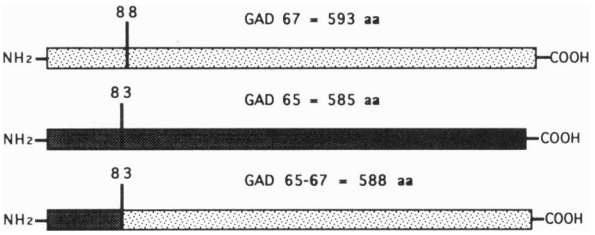

FIG. 2. Diagram illustrating the GAD constructs that were transfected in fibroblasts.

Wild-type CHO cells and GAD-65 and GAD-67 CHO cell clones were grown to confluency in a six-well plate. After pretreatment with 10 mM sodium butyrate for 24 hr, cells were directly solubilized in 200 μl of 1× Laemmli buffer (27) and boiled for 3 min. Equal volumes of the total cell homogenates (35 μl) were separated on SDS/8% polyacrylamide gel and immunoblotted as described (27, 28).

Immunoreactivity in Western blots was detected by enhanced chemiluminescence detection according to the manufacturer's instructions (Amersham). Rat brain and cell line homogenates for Western blotting were prepared as described (14, 24). Protein concentrations were determined with the BCA reagent (Pierce).

Immunofluorescence. Single and double immunofluorescence were performed as described (24). In some experiments, cells were reacted with fluorescein isothiocyanate-conjugated lentil lectin (*Lens culinaris*) (EY Laboratories) after GAD immunostaining.

RESULTS

Stable Cell Lines with Distinct Expression of GAD-65 and GAD-67. To study the intrinsic targeting properties of each of the two GAD isoforms, we developed cloned lines of CHO cells permanently transfected with rat GAD-65 (GAD-65 CHO cells) and GAD-67 (GAD-67 CHO cells). Independent expression of the two GAD isoforms was demonstrated by Western blotting using an antiserum (no. 7673) that recognizes both proteins. As shown in Fig. 3, GAD-65 and GAD-67 expressed in CHO cells had the same electrophoretic mobility as the two major GAD bands in a rat brain homogenate. A higher molecular weight band, which is known to be a GAD dimer (29), was present in brain but poorly represented in the two transfected cell lines (Fig. 3).

In pancreatic islet homogenates, GAD-65, but not GAD-67, was partially recovered in a high-speed pellet and further enriched in the detergent phase after Triton X-114 phase separation (10). Similar distinct properties were displayed by GAD-65 and GAD-67 in transfected cells. Virtually all GAD-67 remained in the supernatant after high-speed centrifugation (Fig. 4*A*). Under the same experimental conditions, the transferrin receptor, an intrinsic membrane protein, was quantitatively recovered in the pellet (Fig. 4*C*). In contrast, GAD-65 was recovered in both the soluble and the particulate fractions (Fig. 4*B*). Upon Triton X-114 extraction and phase condensation, all soluble GAD-67 partitioned in the aqueous phase. Conversely, both soluble and pelletable GAD-65 partitioned in roughly equal amounts in the aqueous and detergent phases. Note also that the transferrin receptor, which has a single transmembrane region (30), partitioned equally in the aqueous and detergent phases (Fig. 4*C*).

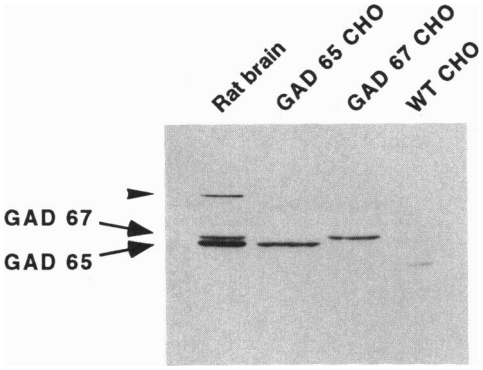

FIG. 3. Expression of GAD-67 and GAD-65 in GAD-65 and GAD-67 CHO cells. Western blots of total tissue (10 μg) and cell homogenates (35 μl) as indicated. WT CHO, wild-type untransfected cells. Immunolabeling was performed with antiserum 7673, which recognizes both GAD isoforms. Mobility of the two GAD bands in CHO cells corresponds to their mobility in brain. A higher molecular weight band visible in brain (arrowhead) is known to represent a GAD dimer.

GAD-65, but Not GAD-67, Is Concentrated in the Area of the Golgi Complex. Immunofluorescence staining of stable transfected CHO clones revealed that GAD-67 was diffusely distributed throughout the cytoplasm, consistent with a cytosolic localization (Fig. 5). By contrast, GAD-65 was highly concentrated in the perinuclear region (Fig. 5). In addition, finely punctate GAD-65 immunoreactivity was observed throughout the cytoplasm. Similar distinct distributions of GAD-65 and GAD-67 were observed in transiently transfected CHO and COS-7 cells (data not shown). No GAD immunoreactivity was detected in wild-type CHO cells stained with antibodies directed against either of the two GAD isoforms (Fig. 5).

Perinuclear localization of GAD-65 was demonstrated to correspond to the Golgi complex by double labeling with Golgi markers—lentil lectin (31) and antibodies directed against α-mannosidase II (17) (Fig. 6). A similar colocalization of GAD and α-mannosidase II was obtained in the β-cell-derived βTC3 cells, which constitutively express the protein (Fig. 7). In addition, in these β cells a large fraction of GAD was colocalized with the synaptic vesicle protein synaptophysin at the cell periphery (6). The perinuclear accumulation of GAD-65 in CHO cells did not colocalize with transferrin receptor immunoreactivity. This result distinguishes GAD-65 from synaptophysin, which, when expressed in CHO cells, is targeted to the same perinuclear vesicles that are positive for transferrin receptor (24). It suggests an association of GAD with elements of the Golgi proper rather than with vesicles that recycles between the Golgi/centrosomal area and the cell periphery.

The different distribution of transferrin receptor and GAD-65 in CHO cells was confirmed by the further dissociation of the two proteins after brefeldin A treatment (Fig.

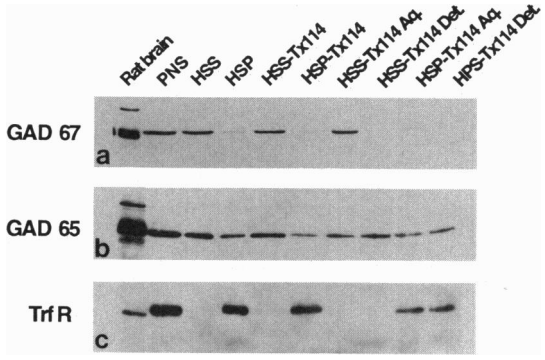

FIG. 4. Distribution of GAD-67, GAD-65, and transferrin receptor (TrfR) from stably transfected CHO cells in subcellular fractions and in detergent and aqueous phases after Triton X-114 phase separation. Lane 1, Immunoblot analysis of various fractions and of total brain homogenate loaded as a reference. GAD-67 and GAD-65 were labeled with antiserum 7673. PNS, postnuclear supernatant; HSS, high-speed supernatant; HSP, high-speed pellet; HSS-Tx114 and HSP-Tx114, Triton X-114 extracts of HSS and HSP fractions. Rightmost four lanes are detergent and aqueous phases of HSS and HSP Triton X-114 extracts as indicated. The same volumes of pellets and supernatants were loaded.

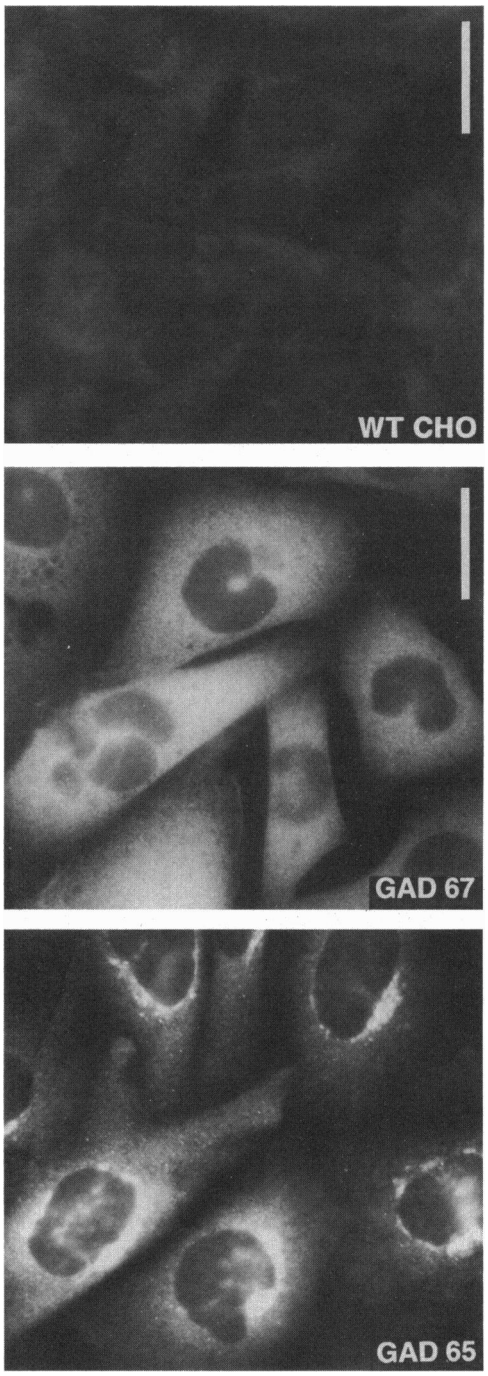

FIG. 5. Differential targeting of GAD-65 and GAD-67 in transfected CHO cells as demonstrated by immunofluorescence. (*Top*) Wild-type CHO cells stained with antiserum 6799. (*Middle*) GAD-67 CHO cells stained with antiserum 6799. (*Bottom*) GAD-65 CHO cells stained with antibody GAD-6. (*Top*, bar = 20 μm; *Middle* and *Bottom*, bar = 12.5 μm.)

6). This drug induces a disassembly of the Golgi complex and a fusion of the transferrin receptor-containing membranes into a tubular network so that localization of a protein in either of these two compartments can be more easily assessed (32). After brefeldin A treatment, the typical GAD-65 perinuclear accumulation was replaced by a diffuse delicate web of cytoplasmic immunoreactivity, which, however, was still clearly distinct from the homogeneous cytosolic pattern of GAD-67. The partitioning of GAD-65 after Triton X-114 extraction was identical before and after brefeldin A treatment (data not shown). Thus, even after brefeldin A treatment, GAD-65 retains its hydrophobic properties and is still associated with subcellular particles.

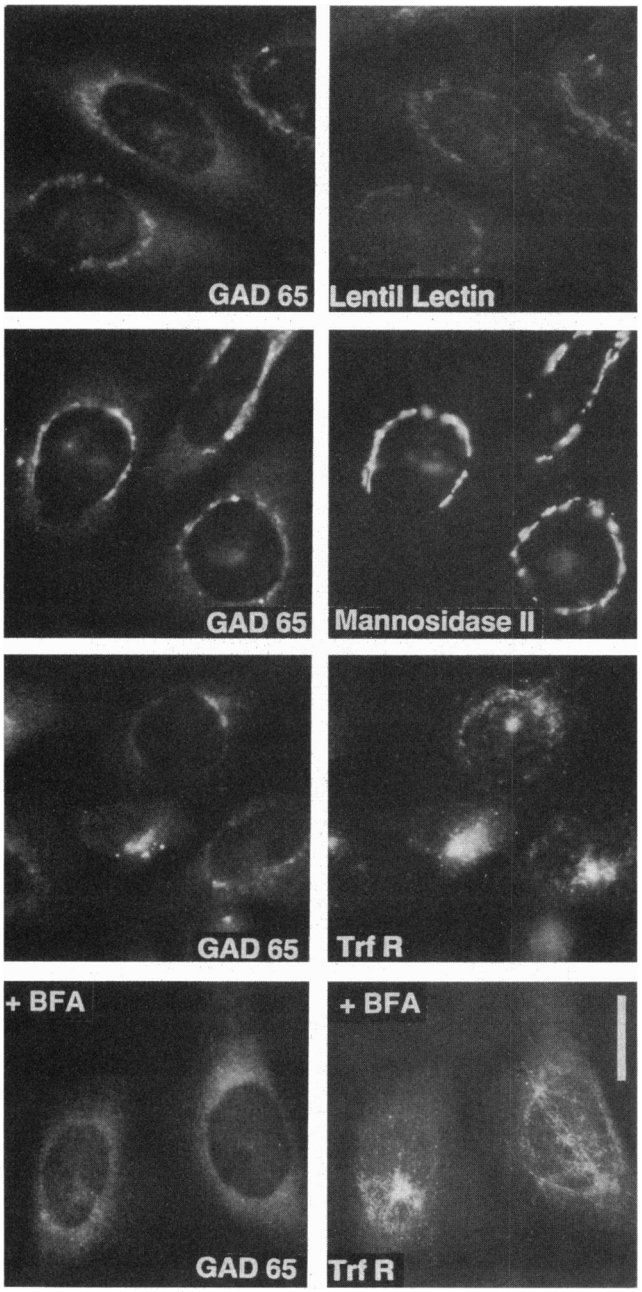

FIG. 6. Double immunofluorescence micrographs showing comparison of distribution of GAD-65 with that of Golgi and early endosomal markers in GAD-65 CHO cells. (*Left*) Immunostaining for GAD-65 with antibody GAD-6 (top two micrographs) and with antibody N65 (bottom two micrographs). (*Right*) Cells were counterstained with lentil lectin, antibodies directed against α-mannosidase II, and antibodies against transferrin receptors (TrfR). Bottom pair of micrographs are from cells treated for 20 min with brefeldin A (BFA) (5 μg/ml). Perinuclear accumulation of GAD immunoreactivity overlaps with that of lentil lectin binding sites and of α-mannosidase II. In contrast, it differs from that of TrfR in both untreated and BFA-treated cells. (Bar = 10 μm.)

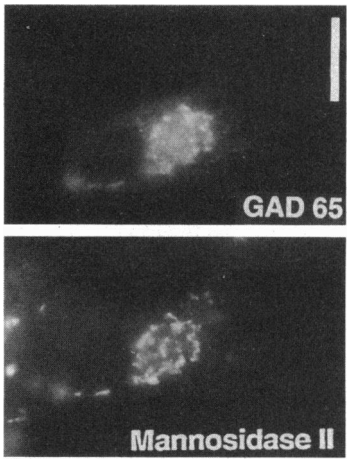

FIG. 7. Double immunofluorescence micrographs showing comparison of distribution of GAD-65 as revealed with GAD-6 antibody and α-mannosidase II in βTC3 cells. Structures visible on the left, which are positive for α-mannosidase II immunoreactivity and negative for GAD-65 immunoreactivity, belong to a cell that does not express GAD. Expression of GAD in βTC3 cells is heterogeneous. (Bar = 7 μm.)

The N-Terminal Region of GAD-65 Contains the Signal for Golgi Complex Association. The targeting of a chimeric GAD-65–GAD-67 protein in which amino acids 1–88 of GAD-67 were replaced by amino acids 1–83 of GAD-65 (Fig. 2) was investigated in stably transfected CHO cells. Despite being mostly composed of GAD-67, the chimeric protein had the same localization observed for GAD-65 (Fig. 8), and its perinuclear accumulation corresponded to the distribution of lentil lectin. This indicates that the first 83 amino acids of GAD-65 contain information required for association with subcellular particles and primarily with the Golgi complex

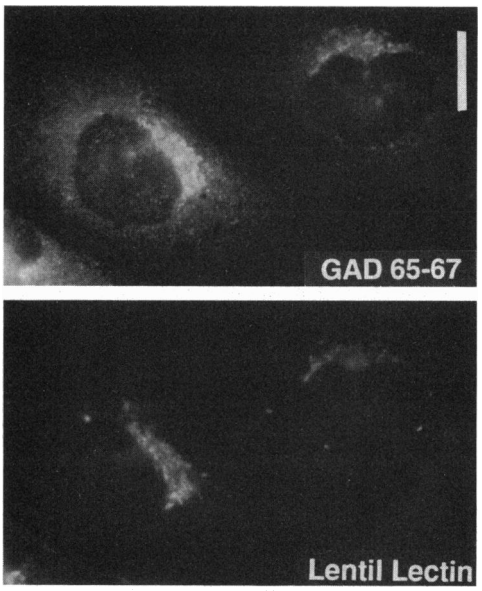

FIG. 8. Localization of chimeric protein GAD-65–GAD-67 in stably transfected CHO cells. Double immunofluorescence showing comparison with distribution of lentil lectin. Perinuclear accumulation of GAD immunoreactivity overlaps with that of lentil lectin. (Bar = 9.5 μm.)

and that they are sufficient to confer this property to the otherwise cytosolic GAD-67.

DISCUSSION

The separate expression of GAD-65, GAD-67, and a chimeric GAD-65–GAD-67 protein in transfected cells indicates that GAD-65 and GAD-67 have different targeting information and that the different intracellular localization of the two proteins is determined by their divergent N termini. The exclusive localization of GAD-67 in the soluble fraction of transfected fibroblasts does not rule out the possibility that in cells expressing both proteins a pool of GAD-67 might be membrane associated via formation of heterodimers with GAD-65. Some evidence suggests the occurrence of GAD heterodimers in the brain (29). Targeting of the two GAD isoforms, when cotransfected in CHO cells, could not be investigated by immunocytochemistry because antibodies specific for GAD-67 were not available. Several antibodies raised against GAD-67 and positive by immunocytochemistry (including the K2 antibody) also recognized GAD-65. In neurons and β cells, GAD, in addition to being localized at the Golgi complex, is concentrated on synaptic vesicles and synaptic-like microvesicles (6). Transfection of GAD constructs in neuroendocrine cells will elucidate whether the associations with the Golgi complex as well as synaptic vesicles and synaptic-like microvesicles are mediated by the same mechanism(s).

Our results complement previous studies that have indicated different biochemical properties of GAD-65 and GAD-67 constitutively expressed in β cells. GAD-65, but not GAD-67, was found to undergo two posttranslational hydrophobic modifications, to partially partition in the detergent phase after Triton X-114 extraction, and to migrate with particulate fractions during high-speed centrifugation (10, 11). Similar properties were reported for GAD-65 expressed in insect cells (11). It was also shown that trypsin digestion of a particulate β-cell fraction containing GAD-65 yielded a 55 kDa C-terminal GAD fragment, which was primarily recovered in the soluble material upon high-speed centrifugation (11). This result suggests a role of the N-terminal region of GAD-65 in membrane association, but an indirect effect of trypsin mediated by the cleavage of a GAD binding protein cannot be excluded. We show here that the N-terminal region of GAD-65 is necessary and sufficient to target the remaining portion of the GAD molecule (the portion highly similar in GAD-65 and GAD-67) to membranous organelles.

Of the two GAD isoforms, at least in stiff-man syndrome, GAD-65 appears to be the dominant autoantigen (16). It is tempting to speculate that the two proteins, possibly due to their different membrane association, may undergo different intracellular processing relevant to their autoantigenic properties.

The precise mechanism by which the N-terminal domain of GAD-65 targets the protein to subcellular particles, and more specifically to the Golgi complex, remains to be elucidated. While the palmitoylation of this domain at cysteine residues may contribute to membrane anchoring (11), other features of the molecules may be involved to account for the specificity of the interaction. In the case of another neuronal peripheral membrane protein, GAP43, palmitoylation was shown to be required for membrane attachment but not sufficient for correct targeting (33).

Pilot studies of CHO cells transfected with GAD-65 constructs mutated at each of the six cysteines contained in the N-terminal region (first 83 amino acids) have suggested that none of these mutations is sufficient to induce a diffuse distribution of GAD-65 similar to that observed for GAD-67 (M.S., R.D., D.A., and P.D.C., unpublished observations). Additional investigations of the N-terminal domain of GAD-65 will establish the role of palmitoylation and of the primary sequence motifs in the targeting of GAD-65.

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