Pancreatic β Cells Express Two Autoantigenic Forms of Glutamic Acid Decarboxylase, a 65-kDa Hydrophilic Form and a 64-kDa Amphiphilic Form Which Can Be Both Membrane-bound and Soluble*

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Stephen Christgau‡§, Helen Schierbeck‡, Henk-Jan Aanstoot‡, Lissi Aagaard‡, Kathi Begley‡, Hans Kofod‡, Kim Hejnæs‡, and Steinunn Baekkeskov‡§
From the ‡Department of Microbiology/Immunology, Department of Medicine, and Hormone Research Institute, University of California San Francisco, San Francisco, California 94143-0534 and the §Hagedorn Research Laboratory, Gentofte, Denmark

The 64-kDa pancreatic β-cell autoantigen, which is a target of autoantibodies associated with early as well as progressive stages of β-cell destruction, resulting in insulin-dependent diabetes (IDDM) in humans, has been identified as the γ-aminobutyric acid-synthesizing enzyme glutamic acid decarboxylase. We have identified two autoantigenic forms of this protein in rat pancreatic β-cells, a M, 65,000 (GAD₆₅) hydrophilic and soluble form of pI 6.6–7.1 and a M, 64,000 (GAD₆₄) component of pI 6.7. GAD₆₄ is more abundant than GAD₆₅ and has three distinct forms with regard to cellular compartment and hydrophobicity. A major portion of GAD₆₄ is hydrophobic and firmly membrane-anchored and can only be released from membrane fractions by detergent. A second portion is hydrophobic but soluble or of a low membrane avidity, and a third minor portion is soluble and hydrophilic. All the GAD₆₄ forms have identical pI and mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results of pulse-chase labeling with [³⁵S]methionine are consistent with GAD₆₄ being synthesized as a soluble protein that is processed into a firmly membrane-anchored form in a process which involves increases in hydrophobicity but no detectable changes in size or charge. All the GAD₆₄ forms can be resolved into two isoforms, α and β, which differ by approximately 1 kDa in mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis but are identical with regard to all other parameters analyzed in this study. GAD₆₅ has a shorter half-life than the GAD₆₄ forms, remains hydrophilic and soluble, and does not resolve into isoforms. Comparative analysis of the brain and β-cell forms of GAD show that GAD₆₅ and GAD₆₄ in pancreatic β-cells correspond to the larger and smaller forms of GAD in brain, respectively. The expression of different forms and their flexiblity in subcellular localization of the GAD autoantigen in β-cells may have implications for both its function and autoantigenicity.

Insulin dependent diabetes mellitus (IDDM) is characterized by a selective loss of the insulin producing β-cells in a process which can span several years, and is characterized by clear indications of an autoimmune response, that includes circulating islet cell antibodies (Castano and Eisenbarth, 1990). We have used the circulating antibodies present in IDDM sera to identify a M, 64,000 (64 Kd) target human islet cell autoantigen by immunoprecipitation of [³⁵S]methionine-labeled human islets (Baekkeskov et al., 1982). Antibodies to the human islet cell 64-kD protein and its rat islet counterpart are present in approximately 80% of newly diagnosed IDDM patients and have been detected up to several years before the clinical onset of the disease, concomitantly with a decrease of β-cell function (Sigurdsson and Baekkeskov, 1990 for review). The 64-kD protein was recently identified as the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) in pancreatic β-cells (Baekkeskov et al., 1990). The presence of GAD and its product GABA in islet β-cells (Garry et al., 1986) and the presence of GABAₐ receptors on islet α- and β-cells (Rorsman et al., 1989; Reusens-Billen et al., 1984) suggests a role of GABA in paracrine signalling between the β-cell and the other endocrine islet cells. GAD is expressed in high concentrations in GABA-ergic neurons in the central nervous system (Mognaini and Oertel, 1985) and in the oviduct (Erdo et al., 1989). Two major forms of GAD have been detected in the brain and their molecular masses have been described as 59–67 kDa (Kaufman et al., 1991; Legay et al., 1987; Chang and Gottlieb, 1988). The larger brain form has been cloned and sequenced from cat (Kaufman et al., 1986; Kobayashi et al., 1987), rat (Julien et al., 1990; Wyborski et al., 1990), and mouse (Katarova et al., 1990). The smaller GAD form in rat brain has recently been cloned, sequenced, and shown to be a product of s different gene (Erlander et al., 1991).

Both the human (Baekkeskov et al., 1987; Baekkeskov et al., 1989) and rat (Christie et al., 1988, 1990) β-cell 64-kD GAD autoantigen have been shown to have amphiphilic membrane protein properties, and this characteristic has been used in most analyses to partially purify the protein. The amphiphilic human β-cell 64-kD GAD autoantigen (GAD₆₄) was detected as two isoforms α and β, which were identical in charge but differed in molecular mass by approximately 1 kDa (Baekkeskov et al., 1989). In the present study, we have...
analyzed autoantigenic forms of GAD in both soluble and membrane compartments of rat islets and characterized those with regard to size, charge, hydrophobicity, and half-life. We show that islets of Langerhans express a less abundant 65-kDa soluble hydrophilic form of GAD (GAD$_{65}$) in addition to GAD$_{90}$, $\alpha$ and $\beta$, and provide evidence that only GAD$_{90}$ becomes membrane bound in a process which involves modification with small noncharged hydrophobic residue(s). We also show that GAD$_{65}$ and GAD$_{90}$ are homologous to the larger and smaller brain forms of GAD, respectively.

**MATERIALS AND METHODS**

Isolation of Islets and Biosynthetic Labeling—Isolation of rat islets, maintenance in culture and radioactive labeling with $[^{35}S]$methionine for 4 h was carried out as described (Baekkeskov et al., 1989). In pulse-chase labeling experiments, rat islets were starved in methionine-free RPMI 1640 medium at $37^\circ$C for 30 min and then given a pulse of $[^{35}S]$methionine (specific activity, $>1000\ $Ci/mmol, Amer- sham) for 30 or 40 min followed by chase periods in medium containing 5 x the normal content of nonradioactive methionine for 0–72 h before harvesting. Labeled islets were harvested by centrifugation, washed five times in isolation buffer containing ice-cold Hapes, pH 7.4, 150 mM NaCl and 10 mM benzamidine/HCl, and then homogenized immediately processed for homogenization and isolation of soluble and membrane compartments or snap frozen and stored in aliquots at $-80^\circ$C.

Analysis of Compartimentalization of the GAD Autoantigen—Islet pellets were suspended in a homogenization buffer, 10 mM Hapes/NaOH, pH 7.4, 0.25% sucrose, 10 mM benzamidine/HCl, 0.1 mM p-chloromercuribenzenesulfonic acid and 0.25% aprotinin (Novo-Nordisk, Bågvaerd, Denmark) (homogenization buffer) at 4°C and separated into crude particulate (P-100) and cytosol fractions (S1-100) by ultracentrifugation at 100,000 x g for 1 h as described (Baekkeskov et al., 1989). The crude P-100 fraction was then washed at 4°C by resuspension in 10 mM Hapes/NaOH (pH 7.4), 150 mM NaCl, 10 mM benzamidine/HCl, 0.25% Trasylol, 0.1 mM p-chloromercuribenzenesulfonic acid and 0.1 mM NaN$_3$V$_3$O$_4$ (Hapes buffer A) followed by ultracentrifugation at 100,000 x g for 1 h resulting in a new S1-100 fraction (S2-100) and a washed P-100 (WP-100) fraction. The WP-100 fraction was extracted in Hapes buffer A containing 2% of either Triton X-100 (TX-100) or Triton X-114 (TX-114) (extraction buffer) at $4^\circ$C for 2 h followed by ultracentrifugation as above.

For quantitative analysis of GAD, the resulting pellet was subjected to repeated extractions and ultracentrifugations. Usually all detergent released in the two first extracts of the WP-100 fraction. The S1-100 and S2-100 fractions and the WP-100 extracts were immunoprecipitated quantitatively with GAD antisera (Christie et al., 1988) either directly or after preincubation with control sera as described (Baekkeskov et al., 1989). In brief fractions were incubated with a mixture of serum fractions which were then isolated by adsorption to excess amounts of protein A-Sepharose (Pharmacia LKB Technology Inc., Uppsala, Sweden). Analysis of supernatants after immunoprecipitation by reimmunoprecipitation with antiserum and protein A-Sepharose revealed that GAD was depleted quantitatively by the first immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. Densitometric scanning of fluorograms was carried out as described (Christie et al., 1988). The results of density measurement of GAD in each fraction were expressed as percent of the sum of density values for GAD in all three fractions. Distribution of total protein into the three fractions was expressed as a percentage of trichloroacetic acid precipitable counts. WP-100 values were expressed as the sum of GAD recovered in repeated extracts of the WP-100 fraction.

For salt and EDTA washing experiments identical aliquots of the WP-100 fraction were taken in either Hapes buffer or the same buffer supplemented with one of the following compositions: 0.5 mM NaCl, 0.2 mM Na$_2$CO$_3$, 0.05 mM NaN$_3$PO$_4$, 0.2 mM MgCl$_2$, 0.05 mM EDTA, or 2% TX-100 and incubated at $4^\circ$C for 1 h. The samples were then ultracentrifuged at 100,000 x g and the resulting supernatants immunoprecipitated and subjected to SDS-PAGE and fluorography.

Immunoblotting—SDS gels were electroblotted to polyvinylidene difluoride membranes (Immobilon, Millipore). Unreacted binding sites were blocked by incubation for 60 min in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 containing 3% skim milk. The blots were incubated for 2 h with the primary antibody diluted in the buffer system of Laemmli (1970). After washing, the blots were incubated for 1 h with a horseradish peroxidase (HRP) conjugated polymers (K2, 1:2000; GAD6 ascites 1:500) followed by visualization with alkaline phosphatase-labeled antibodies. Antisera—The antisera used in this study included three IDDM patient sera which were strongly positive for GAD autoantibodies and three control sera from healthy individuals; a polyclonal rabbit antiserum (1266) generated against a synthetic peptide prepared according to described methods (Aatar et al., 1989) and containing the C-terminal sequence of the larger rat brain form of GAD (Cys-Trp-Ser-Ser-Arg-Tyr-Glu-Leu-Leu-His-Pro-Ile-Leu-Thr-Ser-Ser-Ser-Arg-Arg) (Julien et al., 1990; Wyborski et al., 1990) coupled to keyhole limpet hemocyanin (a gift from Dr. J. S. Petersen, Hagedorn Research Laboratory, Gentofte Denmark); GAD6, a mouse monoclonal antibody (ascites), which was raised against immunooaffinity purified rat brain GAD and which specifically recognizes the smaller GAD form in brain on Western blots (Chang and Gottlieb, 1992) (a gift from Dr. D. Gottlieb, Univ. of Washington, St. Louis), and K2, a rabbit antiserum raised to the larger form of cat GAD produced in a bacterial expression system and which almost exclusively reacts with the larger brain form on Western blots (Kaufman et al., 1991) (a gift from Dr. Allan Tobin, UCLA).

**RESULTS**

Autoantigenic Forms of GAD in Pancreatic $\beta$-Cells

**Immunoblot Expression of Larger Form of Brain GAD in COS CELLS—**The eukaryotic expression vector 91023B (Wong et al., 1985) (a gift by Dr. Randall Kaufman, Genetics Institute, Boston) was modified at the EcoRI cloning site with synthetic linkers to produce a Spel site. This plasmid is called pXPEP130. A plasmid containing a full-length cDNA clone coding for the larger rat brain form of GAD (a gift from Dr. A. Tobin, UCLA) was digested with XbaI to isolate a 3-kb fragment corresponding to nucleotides 250 to 2750. The 5' 200 base pairs are intrinsic (an artifact in cDNA synthesis) but contain
were added to 100-mm dishes of COS7 cells at 80% confluency in protocol. 60 pg of lipofectin and 30 pg of either pGAD16 or pGAD17 using a lipofectin reagent (BRL) according to the manufacturer's particles fractions (Christie et al., 1988, Baekkeskov et al., medium with 20% fetal bovine serum was added. Cells were harvested after 48 h, snap frozen, and stored in aliquots at -80°C.

The α-isoform did not differ from the hydrophilic GAD65 of human and rat GAD, autoantigen. The isoform was also detected as two isoforms, among the preparations (Fig. 1C) and the isoform was immunoprecipitated from rat islets by GAD antibody-positive sera from three IDDM patients (lanes 4–6). Immunoprecipitation with sera from three healthy control individuals is shown in lanes 1–3. [35S]Methionine-labeled rat islets were homogenized in an isotonic Hepes/sucrose buffer and the homogenate centrifuged at 100,000 x g for 1 h. The pellet (P-100) was extracted in Triton X-114 and the lysate subjected to temperature-induced phase separation. The detergent-enriched phase was preclaved with normal human serum and then immunoprecipitated. In addition to the positions of GADα/β, the positions of actin (α) and tubulin (β), which were present as background in all immunoprecipitates, are indicated. The sera used in lanes 4 and 5 were used in all subsequent analyses of GAD and sera in lanes 1 and 2 as control sera. B. NEPHGE/SDS-PAGE analysis of particulate amphiphilic GADα4 prepared as in A. The pl of the α and β components was determined by a separate analysis using IEF/SDS-PAGE (not shown). C, SDS-PAGE analysis of amphiphilic GADα4 immunoprecipitated from either soluble (S2-100) or particulate (WP-100) fractions of three different rat islet cell preparations. The crude particulate fraction (P-100) from each islet preparation was washed by resuspension in Hepes buffer, followed by centrifugation at 100,000 x g for 1 h to prepare a supernatant (S2-100) and a washed particulate fraction (WP-100). TX-114 detergent phase-purified material from each fraction was immunoprecipitated with either a control serum (C) or a GAD antibody-positive IDDM serum (J). The GADα/β ratio varies between the three preparations shown in lanes 1–4, 5–8, and 9–12, respectively, but is similar for S2-100 and WP-100 in each preparation. Note that samples from S2-100 (lanes 5 and 6) and WP-100 (lanes 7 and 8) of one islet preparation were analyzed on two separate gels.

Detection of Amphiphilic and Hydrophilic GADα4 but Only Hydrophilic GADα4 in Soluble Fractions of Rat Islets—To analyze the distribution of the GAD autoantigen into soluble and membrane compartments, islets were homogenized and subjected to ultracentrifugation to prepare a primary cytosol (S1-100) fraction. The crude particulate fraction (P-100) was resuspended in an isotonic Hepes buffer to remove cytosolic proteins and proteins loosely associated to membranes, followed by ultracentrifugation to prepare a secondary cytosol fraction (S2-100) and a washed particulate fraction (WP-100). GAD was immunoprecipitated from the different fractions using GAD antibody-positive IDDM sera and analyzed by SDS-PAGE and fluorography.

Analyses of the soluble fractions of S1-100 and S2-100 showed that the expression of GAD in rat islets was not restricted to membrane bound compartments. Thus SDS-PAGE (Fig. 2, A and B) and 2D gel electrophoresis (Fig. 2C) showed the presence of the GADα4 form of pl 6.7 in both the S1-100 and S2-100 cytosol fractions in addition to the particulate fractions. No difference was detected in the mobility of GADα4 in the different compartments (Fig. 1C; Fig. 2). The analysis of the soluble and particulate fractions furthermore revealed an additional form of GAD of M, 65,000 and pl 6.9–

7.1 (GADβ) in the S1-100 and S2-100 fractions (Fig. 2A, lane 2; Fig. 2C, a and c). GADβ was absent in the WP-100 fraction (Fig. 2C, d). Electron microscopic analysis of the S1-100 and S2-100 fractions did not reveal the presence of small membrane vesicles or fragments (data not shown), suggesting that the GADα4 and GADβ forms detected in these fractions were indeed soluble.

To analyze the hydrophobicity of GADα4 and GADβ in the different fractions, the soluble fractions S1-100 and S2-100 and the particulate fractions were subjected to TX-114 phase separation. The distribution of GADα4 and GADβ into the detergent and aqueous phases was analyzed by immunoprecipitation. This analysis showed that GADβ was only present in the aqueous phase and never partitioned into the detergent phase demonstrating that this form is hydrophilic (Fig. 2, A and B). However, whereas GADα4 in both the S2-100, P-100, and WP-100 fractions partitioned into the TX-114 detergent phase (Fig. 2, A and B), GADα4 in the S1-100 fraction was...
detected predominantly in the aqueous phase (see below), suggesting heterogeneity in hydrophilic/hydrophobic properties of GADα in the different compartments.

We considered that the difference in mobility between the α and β components of GADα might represent the membrane anchor, and therefore that the distribution of those components might differ between the soluble and particulate compartments. The α/β ratio was however identical in each compartment (Fig. 1C; Fig. 2B and results not shown). In the preparations where the β isoform was not evident, the α isoform was still found in the different compartments (Fig. 2, A and C). Thus the α and β isoforms of GADα show identical behavior with regard to compartmentalization.

As described earlier (Baekkeskov et al., 1988, 1989) TX-114 detergent phase purification of the amphiphilic form of GAD54 efficiently eliminates background proteins in immunoprecipitates (Figs. 1 and 2). In contrast, several background proteins can be detected in immunoprecipitates of crude cellular fractions and in particular in the aqueous phases using either IDDM or control sera (Fig. 2A, lanes 2–5; Fig. 2B, lanes 3, 4, 6, 7). As shown elsewhere (Baekkeskov et al., 1989) the background proteins represent a minor fraction of abundant cellular proteins carried nonspecifically through the immunoprecipitation procedure. Since GAD is a very rare protein in the soluble cellular proteins carried nonspecifically through the immunoprecipitation procedure. Since GAD is a very rare protein in the soluble and particulate compartments. The values presented in Fig. 3, ~55% in S1-100 and ~40 in S2-100, and 5% in WP-100 are therefore a

\[ \text{Fig. 2. One- and two-dimensional gel electrophoretic analysis of GAD in membrane bound and soluble compartments. A, SDS-PAGE analysis of GAD immunoprecipitated from S1-100 and P-100 fractions. Primary cytosol (S1-100) and a crude particulate (P-100) fraction were prepared and detergent phase (d) and aqueous phase (a) derived from the P-100 extract as described in Fig. 1, followed by immunoprecipitation with control (C) and IDDM (I) sera, SDS-PAGE analysis using modified Laemmli buffer, and fluorography. The immunoprecipitates in lanes 2 and 3 represent S1-100 prepared from 3000 rat islets and the immunoprecipitates in lanes 4–7 represent P-100 prepared from 1000 islets to obtain comparable signal intensities between the soluble S1-100 and particulate P-100 fractions. In addition to GADα, the IDDM serum specifically immunoprecipitates a 65-kDa band (GADα) from S1-100 (lane 2). This band is also present in the aqueous phase of the P-100 fraction (lane 4), although it is not clearly distinguished from the background. Only GADα is present in the detergent phase of the particulate fraction (lane 6). M, markers at the positions indicated are shown in lane 1. The M marker with mobility between the GADα and GADβ α (indicated with an arrowhead in lane 1) and the background band indicated with an arrowhead in lane 3, which has been shown to have mobility in between the GADα α and β components, were used together with the TX-114 phase distribution pattern to identify the two bands as GADα and GADβ α as opposed to GADα α and β. 2D analyses confirmed that this preparation of islets lacked the GADβ α (not shown). The background bands in immunoprecipitates of S1-100 and the aqueous phases represent a small fraction of major cellular proteins carried nonspecifically through the immunoprecipitation procedure. IDDM sera may, however, specifically recognize a weak 55-kDa band in lanes 2 and 4 (see legend to Fig. 2B); B, SDS-PAGE analysis of GAD immunoprecipitated from aqueous and detergent phases prepared from S2-100 and WP-100 fractions as described in the legend to Fig. 1B. M, markers are shown in lane 5. GADα is absent in WP-100 (lanes 7 and 9) but present in S2-100 aqueous phase (lane 3). GADα does not partition into the TX-114 detergent phase (lane 2). In addition to GADα and GADβ α, the IDDM serum specifically immunoprecipitates a 55-kDa band (open arrow) from the aqueous phases (lanes 3 and 7). This protein may represent a proteolytic fragment of GADα or GADβ α. C, NEPHGE/SDS-PAGE analysis of immunoprecipitates of S1-100 with IDDM serum (a) and a control serum (b) and of S2-100 (c) and WP-100 (d) with IDDM serum. The exact plS of the GADα and GADβ α/β components were}

\[ ^{1} \text{K. Hejnaes and S. Baekkeskov, unpublished results.} \]
\[ ^{2} \text{S. Christgau and S. Baekkeskov, unpublished observation.} \]
brane-bound islet cell compartments. Portions of cellular proteins, of HEPES buffer A and ultracentrifuged to yield S2-100 and WP-100 fractions. S1-100, S2-100, and P-100 extracts were precleared twice by immunoprecipitation with normal serum and then divided in two aliquots and immunoprecipitated with GAD antibody-positive IDDM serum and a negative control serum, followed by resolution on SDS-gels in modified Laemmli buffers and fluorography. The distribution of GADβ3 (dark-shaded bars), GADε4 (shaded bars), and total cellular proteins (open bars) into the three fractions was assessed as described under "Materials and Methods" and mean ±S.D. calculated from six experiments.

rough estimate. Nevertheless they demonstrate that GADβ3 is soluble and not membrane-anchored. Thus rat islets express two forms of GAD recognized by autoantibodies in IDDM, a soluble and hydrophilic form, GADβ3, of pI 6.9–7.1 and a second form, GADε4, of pI 6.7 which is heterogeneous with regard to both subcellular localization and hydrophobicity.

A Major Portion of GADε4 Is Firmly Membrane-anchored

The results described above demonstrate that a subpopulation of GADβ3 behaves as a soluble protein during traditional cell fractionation, and suggested that membrane-bound GADε4 might not be an integral membrane protein, but rather associated with the periphery of the membrane, such that it can be released during washing of the particulate fraction. To address this possibility, compounds which release peripheral membrane proteins were tested for their ability to release GADε4. Aliquots of WP-100 fractions were incubated in Hepes buffer A supplemented with one of the following compounds: 0.5 M NaCl; 0.2 M Na2CO3 (pH 10.9); 0.5 M Na2P2O7; 0.2 M MgCl2; or 50 mM EDTA. In addition, the detergent TX-100 was used to release integral membrane proteins. After ultracentrifugation to remove insoluble material, the supernatants were analyzed for released proteins by immunoprecipitation and SDS-PAGE (Fig. 3). GADε4 in the WP-100 fraction was only released from the membranes in a significant manner by detergents, but not by any of the agents known to release peripheral membrane proteins. This result indicates that the hydrophobic GADε4 exists in two different forms with regard to cellular compartments. One form, found in the S2-100 fraction, is soluble or has a low membrane avidity. The other form, found in the WP-100 fraction, is firmly membrane-anchored and, in the presence of enzyme inhibitors (Hepes buffer A), can only be released from the membrane by detergent.

Time-dependent spontaneous release of membrane anchored GADε4 from the WP-100 fraction can, however, take place in buffer compositions without enzyme inhibitors and may signify an endogenous enzyme capable of removing the membrane anchor of GADε4 and resulting in the amphiphilic but soluble S2-100 form.

Differential Hydrophobicity of the Membrane-bound and Soluble Forms of GADε4

A portion of GADβ3 consistently remained in the aqueous phase even after repeated detergent extractions and phase separations. This distribution pattern might either be due to its size precluding quantitative partitioning into the detergent phase (Bjerrum et al., 1983), or to a heterogeneity in the protein with regard to hydrophobic properties. As shown above, a large proportion of GADβ3 in both the S2-100 and WP-100 fractions partitioned into the TX-114 detergent phase. To investigate whether the soluble and membrane-bound fractions of GADβ3 differed in their amphiphilic properties, we analyzed their distribution between the two phases. The S1-100, S2-100, and WP-100 fraction were each subjected to a phase separation using identical buffer compositions in the three fractions. The distribution of GADβ3 in the aqueous phase and detergent phase was analyzed by quantitative immunoprecipitation and SDS-PAGE. The relative quantity of GADβ3 in the different phases was estimated by densitometric scanning of fluorograms from nine experiments and compared to the distribution of total cell proteins (Fig. 3). In all the experiments, no differences in the ratio between the α and β forms of GADβ3 were detected between the aqueous and detergent phases. In the nine independent experiments, total cellular proteins in the S1-100 and S2-100 fractions behaved similarly, with ~10% and ~11% distributing into the detergent phase, respectively. As expected, a much higher proportion, ~55% of the total cellular proteins in the WP-100 fraction separated into the detergent phase. The corresponding figures for GADε4 in the detergent phases, as assessed after immunoprecipitation, were ~8% in the S1-100, and ~60% in the WP-100 fractions, respectively. The results show that the membrane-anchored and S2-100 fraction...
forms of GAD6a are more hydrophobic than GAD6b found in the S1-100 cytosol fraction. Furthermore, the membrane-anchored GAD6a and the S1-100 GAD6b each follow the general pattern of TX-114 partition characteristics in their respective compartments. The hydrophobic characteristics of GAD6a in the S2-100 fraction were, however, anomalous for that fraction, in that ~50% partitioned into the detergent phase. GAD6b residing in this fraction is either cytosolic or has been released from the membrane, and yet displays a TX-114 binding pattern resembling that of the protein in the particulate fraction. The data are compatible with the existence of three populations of GAD6b, which differ with regard to compartment and hydrophobicity. One form is localized to the S1-100 fraction and is mainly hydrophilic. The second form, which is most concentrated in S2-100, has a significantly increased hydrophobicity compared to the S1-100 form and seems to have a low membrane avidity. The third form, which predominates in the WP-100 fraction, has similar hydrophobicity as the S2-100 form but differs in being tightly membrane-bound.

**Pulse-chase Analysis of GADb and GADDa**—Islets were labeled for short periods and then subjected to chase periods of 1–48 h to assess the half-life of GADDa and GADDa in soluble and membrane-bound compartments. The results of one such experiment are shown in Fig. 6. Based on densitometric scanning of the autoradiogram shown in Fig. 6 and of three other experiments it was concluded that α and β of GADDa had the same rate of turnover in each individual fraction, with the maximum incorporation at 4 h of chase. In contrast, GADDa had a maximum incorporation at 0 h of chase, and had a shorter half-life (<4 h) than the GADDa α/β doublet. GADDa was hydrophilic throughout the time course in that it did not separate into the detergent phase (data not shown). The half-life of GADDa in the three different fractions S1-100, S2-100, and WP-100 was estimated to be about 6–10 h in the S1-100 fraction, about 22–28 h in the S2-100 fraction, and about 20–30 h in the WP-100 fraction. TX-114 phase separation of pulse-chase-labeled material showed that GADDa in the S1-100 and S2-100 fractions displayed the same amphility pattern as demonstrated in the 4-h-labeled islets, i.e. the form predominating in S1-100 being hydrophilic and the S2-100 and WP-100 forms being amphiphilic (about 50% distributing into the detergent phase, data not shown). These results suggest that the GADDa autoantigen in the β-cells is synthesized as a hydrophilic soluble form which predominates in the S1-100 fraction and then is processed into the hydrophobic forms seen in the S2-100 and WP-100 fractions by a maturation process that results in membrane anchoring for the WP-100 form.

**Comparative Analysis of Brain and β-Cell Forms of GAD**—We have shown that brain and β-cell forms of GAD have identical mobility by SDS-PAGE and identical patterns on two-dimensional gels (NEPHGE/SDS-PAGE) Baekkeskov et al., 1990), suggesting that islets and brain express identical forms of GAD and that GADDa and GADDa in islets correspond to the larger and smaller forms of GAD in brain (Chang and Gottlieb, 1988), respectively. The smaller brain form has been cloned and sequenced recently and shown to be encoded by a different gene than the larger form (Erlander et al., 1991). Thus in brain the two forms are clearly different and do not have a precursor-product relationship. To further assess the possible identity between the GAD forms in the two tissues, the brain and β-cell forms of GAD, as well as the larger brain form transfected and expressed in COS cells, were analyzed in parallel by immunoblotting using a set of distinctive antibodies: 1) An antibody (1266) raised against a C-terminal peptide in the larger brain form, which recognizes both forms of GAD in brain (Fig. 7, lane 5), in agreement with the homology between the two forms at the C terminus (Erlander et al., 1991); 2) an antibody which preferably recognizes the larger brain form (the K2 antibody, Kaufman et al., 1991); or 3) an antibody which is specific for the smaller brain form (the GAD6 antibody, Chang and Gottlieb, 1988). As shown previously (Baekkeskov et al., 1990) the mobility of GADDa and GADDa in islet cells on SDS-PAGE was identical to that of the larger and smaller brain forms of GAD respectively (Fig. 7). The 1266 antibody recognized both GADDa and GADDa in β-cells (Fig. 7, lane 6). The K2 antibody specifically stained GADDa in islets (Fig. 7, lane 3) in analogy with its staining of
the larger brain form of GAD in both the COS expression system and brain tissue (Fig. 7, lanes 1 and 2). In contrast GAD_{64} and the smaller brain form were specifically recognized by the GAD6 antibody which did not stain GAD_{65} or the larger brain form (Fig. 7, lanes 7–9). Those results are consistent with GAD_{65} and GAD_{64} representing two distinct forms of GAD in pancreatic β-cells which are identical in size and antigenicity to the larger and smaller brain forms of GAD, respectively.

**DISCUSSION**

The 64-kDa autoantigen, which is a major target of autoantibodies associated with insulin dependent diabetes has been identified as the GABA-synthesizing enzyme glutamic acid decarboxylase in pancreatic β-cells (Baekkeskov et al., 1990). In the present report we have used human autoantibodies to identify and characterize the different autoantigenic forms of this enzyme in membrane-bound and soluble fractions of pancreatic β-cells in steady state and pulse-chase-labeling experiments. Although GAD has been detected in both membrane bound and soluble compartments of brain (Chang and Gottlieb, 1988; Baekkeskov et al., 1990) and β-cells (Baekkeskov et al., 1990), this study represents the first detailed characterization of membrane bound and soluble forms of this enzyme. The results show that pancreatic β-cells express two distinct forms of GAD, a larger form of M_{r} approximately 65,000 (GAD_{65}), which is hydrophilic and soluble and has a pI of 6.9–7.1, and a smaller form of M_{r} approximately 64,000, and pI 6.7 (GAD_{64}), which partitions between soluble and membrane bound compartments and is heterogeneous with regard to amphiphilicity. The pulse-chase analysis suggests that both GAD_{65} and GAD_{64} are synthesized as hydrophilic soluble molecules, which are predominantly found in the S1-100 fraction, and that only GAD_{64} is posttranslationally modified to become an amphiphilic molecule which can either be soluble or firmly membrane anchored. It is conceivable that GAD_{64} is modified by hydrophobic residues in a two-step process, which results in first a hydrophobic form which is either soluble or of a low membrane avidity and which is predominantly found in the S2-100 fraction and second, a firmly membrane-anchored form found in the WP-100 fraction. The second step may be reversible. The modification of GAD_{65} is not accompanied by detectable changes in size or charge, suggesting that the modification is mediated by small hydrophobic noncharged residues. Based on those results we propose that the membrane anchoring is mediated by a small lipid or fatty acid(s).

2D gel electrophoretic analysis of the GAD forms in rat brain and islets showed that both tissues have a larger and a smaller GAD form of similar size and pI (Baekkeskov et al., 1990). Based on the 2D gel electrophoretic analysis (Baekkeskov et al., 1990) and the size and immunochemical comparisons of the brain and β-cell forms presented here, we conclude that the 65- and the 64-kDa β-cell form correspond to the larger and smaller brain forms of GAD, respectively. We have recently isolated cDNA spanning the entire amino acid coding region for GAD_{65} in rat islet cells. Sequencing of the cDNA confirmed that the sequence of GAD_{65} in islets is identical to that of the larger GAD form in rat brain (Julien et al., 1990; Wyborski et al., 1990). The amino acid sequence of the GAD_{65} form does not contain membrane anchoring domains (Julien et al., 1990; Wyborski et al., 1990) in agreement with the soluble hydrophilic properties of GAD_{65} demonstrated in the present study. Similarly the amino acid sequence of the smaller rat brain form of GAD does not contain stretches of hydrophobic amino acids (Erlander et al., 1991), in agreement with our results that GAD_{64} in islets is also being synthesized as a hydrophilic soluble molecule. The amphiphilic properties of the more mature GAD_{64} and the ability of this form to become membrane anchored, however, clearly distinguishes it from GAD_{65}, which remains hydrophilic and soluble throughout its lifetime.

What is the subcellular localization of GAD? In brain, electron microscopic studies suggest that GAD is present in proximity to or associated with the membrane of synaptic vesicles which contain GABA (Wood et al., 1976). Immunogold electron microscopic analysis of pancreatic sections suggest that GAD is localized to the membrane of small vesicles in β-cells, which contain GABA and stain for the synaptic vesicle protein synaptophysin (Aanstoot et al., 1991; Reetz et al., 1991). It is thus conceivable that membrane bound GAD may become visible at the surface following fusion of GABA-containing vesicles with the plasma membrane during secretion. However the exact subcellular distribution of the two forms of GAD in brain and β-cells remains to be elucidated. The two forms of mammalian GAD are encoded by two distinct and unlinked genes (Erlander et al., 1991). The reason why two different forms of GAD have evolved is unknown. It is conceivable that the differences in compartmentalization of the two GAD forms demonstrated in this study may affect their enzymatic functions and influence the intracellular routes of transport and secretion of their product, GABA. Furthermore the ability of the smaller β-cell form to be either membrane bound or soluble may reflect the possibility to control its amount in membrane compartments, a characteristic which may influence the visibility of the protein to the immune system.

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