Expression of the Vesicular Inhibitory Amino Acid Transporter in Pancreatic Islet Cells

Distribution of the Transporter Within Rat Islets

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γ-Aminobutyric acid (GABA) is stored in microvesicles in pancreatic islet cells. Because GAD65 and GAD67, which catalyze the formation of GABA, are cytoplasmic, the existence of an islet vesicular GABA transporter has been postulated. Here, we test the hypothesis that the putative transporter is the vesicular inhibitory amino acid transporter (VIAAT), a neuronal transmembrane transporter of GABA and glycine. We sequenced the human VIAAT gene and determined that the human and rat proteins share over 98% sequence identity. In vitro expression of VIAAT and immunoblotting of brain and islet lysates revealed two forms of the protein: an ~52-kDa and an ~57-kDa form. By immunoblotting and immunohistochemistry, we detected VIAAT in rat but not human islets. Immunohistochemical staining showed that in rat islets, the distribution of VIAAT expression parallels that of GAD67, with increased expression in the mantle. GABA, too, was found to be present in islet non–β-cells. We conclude that VIAAT is expressed in rat islets and is more abundant in the mantle and that expression in human islets is very low or nil. The rat islet mantle differs from rat and human β-cells in that it contains only GAD67 and relatively increased levels of VIAAT. Cells that express only GAD67 may require higher levels of VIAAT expression. *Diabetes* 51:1763–1771, 2002

Expression of GAD is a characteristic of the islets of Langerhans. GAD is produced in a number of different tissues but is most abundant in brain and pancreatic islets (1). There are two major isoforms of the enzyme: GAD65 and GAD67. For unknown reasons, islet expression of the two isoforms differs markedly in different animals. Rat islets express both GAD65 and GAD67. In contrast, human islets contain only GAD65 (2); GAD67 protein is not detectable, and GAD25, a nonenzymatically active GAD67 splice variant (3), is present in a relatively sparse subset of cells (S.D.C., W.T.S., unpublished observations) (2,4).

GAD65 and GAD67 catalyze the formation of the neurotransmitter γ-aminobutyric acid (GABA) from glutamate. The role of GABA in the islet is unclear; there is evidence that points to roles as a paracrine regulator of glucagon and somatostatin release, as a metabolic intermediary, and as an inhibitor of first-phase insulin release (4–6). GAD65 is a major autoantigen in type 1 diabetes (2).

GAD65 is primarily membrane-associated and targeted to the cytoplasmic surface of the synaptic-like microvesicle (SLMV), a secretory organelle found in endocrine cells that is the counterpart of the neuronal synaptic vesicle (7). GAD67 associates to a lesser extent with the SLMV; it is mostly distributed homogeneously throughout the cytosol (8).

GABA accumulates within the SLMV, from which it is probably secreted in its role as a signaling molecule (6,9). Because neither GAD65 nor GAD67 is a transmembrane protein, another protein likely mediates GABA entry into the SLMV. Experiments using isolated microvesicles from a mouse β-cell line have provided evidence of such a SLMV GABA transporter. The microvesicles displayed a GABA transport activity that depended on a proton electrochemical gradient (10). Acidification of the SLMV lumen depended on the functioning of an ATP-driven proton pump. Interestingly, an electrochemical gradient also promotes phosphorylation of GAD65, increasing its enzymatic activity (11). GABA production and transport, then, may be regulated in parallel.

A vesicular transporter of GABA and glycine has been identified in mouse and rat brain: the vesicular inhibitory amino acid transporter (VIAAT) (12,13). VIAAT transports GABA and glycine into acidic vesicles and localizes to the synaptic vesicle in glycinergic and GABAergic neurons (13,14). Mouse and rat VIAAT are 98% identical.

To better understand how GABA enters the SLMV before secretion, we desired to identify the islet cell GABA transporter. Because of the similarity between the synaptic vesicle and the SLMV and because VIAAT and the putative β-cell transporter both require an electrochemical gradient to function, we reasoned that VIAAT was a good candidate for the islet transporter (12,15). To study VIAAT expression in human islets, we first sequenced the human gene. Our goals were to determine whether VIAAT is expressed in islets, to characterize its distribution, and to ask whether expression varies between human and rat islets.

RESEARCH DESIGN AND METHODS

Analysis, cloning, and expression of human VIAAT cDNA. A candidate human VIAAT gene was identified by using BLAST (National Center for

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Received for publication 19 July 2001 and accepted in revised form 21 February 2002.

GABA, γ-aminobutyric acid; hVIAAT, human vesicular inhibitory amino acid transporter; SLMV, synaptic-like microvesicle; VGAT, vesicular GABA transporter; VIAAT, vesicular inhibitory amino acid transporter.

Biotechnology Information) to search for human genomic sequences homologous to the rat gene (16). Genomic data were submitted to GENSCAN (Massachusetts Institute of Technology, Boston, MA) for gene prediction. VIAAT was amplified from human brain cDNA (OriGene Technologies, Rockville, MD) by PCR using flanking primers derived from the candidate sequences: 5′-CTCGGGTCCTTCTGTCCTT and 3′-AGAAGGGAGAGAGCGCAGA. Internal primers were used for a second round of amplification: 5′-GCCGCCATGGCCACCTTGCTC and 3′-CGGGATCCTTGCGCCCTAGTCCTC. PCR products were analyzed by agarose gel electrophoresis and sequenced using the ABI PRISM BigDye Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). NetPhos 2.0 (17) and the protein secondary structure prediction software PHD (18) were used for prediction of protein phosphorylation sites and transmembrane regions.

For in vitro expression, the human VIAAT (hVIAAT) cDNA was cloned into the plasmid pCRII-TOPO (Invitrogen, Carlsbad, CA) downstream of the plasmid's Sp6 promoter. GAD65 was expressed as previously described (19). A rabbit reticulocyte lysate system (Promega, Madison, WI) and canine pancreatic microsomal membranes (Promega) were used for in vitro transcription and translation experiments, following the manufacturer's instructions. The quality of the microsomal membrane preparation was verified by assaying glycosylation and signal peptidase activities using transcripts and a protocol provided by the manufacturer.

Antibodies. An affinity-purified peptide antibody to the COOH-terminal 17–amino acid residues of rat VIAAT (COOH-terminal antibody) and a sample of the immunizing peptide were purchased from Chemicon (Temecula, CA). An antibody generated against the NH_2-terminal 127 residues of mouse VIAAT (NH_2-terminal antibody) was a gift from Dr. Bruno Gasnier (20). Antibody vesicular GABA transporter (VGAT)/1 (21) to rat VIAAT residues 75–87 was from Synaptic Systems (Göttingen, Germany). Antibody 7309 is a peptide antibody against the NH_2-terminus of GAD65 (22). The antibody to the NH_2-terminus of GAD67, 9886, was generated in parallel with the previously described antibody, 11616, using the same peptide and methodology and exhibits the same specificity for GAD67 (22). A polyclonal antibody to glucagon was purchased from Zymed Laboratories (South San Francisco, CA). Monoclonal antibodies to glyceraldehyde-3-phospate dehydrogenase, to the COOH-terminus of GAD65 and GAD67 (GC-3108), and to GABA (GB-69) were obtained from Chemicon, from Affiniti Research (Exeter, U.K.), and from Sigma (St. Louis, MO), respectively.

Immunostaining. Pancreata were taken from either healthy diabetes-resistant (DR) BB rats or diabetic diabetes-prone (DP) BB rats and paraformaldehyde-fixed (23). Rats were 150 days old; DP rats became diabetic at 63–70 days and were insulin-treated thereafter. Normal adult human pancreas tissue was provided by the Cooperative Human Tissue Network (Cleveland, OH). Tissue was sectioned (5 μmol/l), deparaffinized, rehydrated, and blocked in PBS with 1% BSA and 2% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. The serum was heat-treated, which prevents nonspecific complement-mediated antibody binding to islet cells (24). An avidin/biotin blocking kit (Vector Laboratories) was used per the manufacturer's instructions. The primary antibody was diluted in blocking solution and applied to the sections overnight at 4°C. Primary antibody dilutions were as follows: COOH-terminal VIAAT, 10 μg/ml; anti-GAD67, 1:200; anti-GAD65, 1:200; NH_2-terminal VIAAT, 1:200; VGAT/1, 1:1,000; anti-glucagon, 1:100; and anti-GABA, 1:100. A 30-min room temperature incubation in a 1:500 dilution of biotinylated goat anti-rabbit IgG followed by a 30-min incubation in a 1:200 dilution of alkaline phosphatase streptavidin and development with Vector Red or NBT/BCIP substrate (all from Vector Laboratories) were used to visualize the binding of the primary antibodies. To help test specificity, before use, the COOH-terminal antibody was sometimes diluted to 10 μg/ml in PBS with 1% BSA and an ~80-fold molar excess of the immunizing peptide or an irrelevant control peptide. The University of Washington Diabetes and Endocrinology Research Center (DERC) Immunohistochemistry Core performed the immunostaining shown in Fig. 7.

Protein samples. Human and rat brain protein extracts were purchased from Chemicon and Clontech Laboratories (Palo Alto, CA). Human islets (~70–80% pure) were provided by the Human Islet Transplantation in Seattle program (Seattle, WA). Islets from healthy DR BB rats were provided by the Islet Satellite of the University of Washington DERC Cell and Tissue Core. Human and rodent islets were harvested after Liberase-mediated pancreas digestion (Roche Molecular Biochemicals, Indianapolis, IN). Liberase was injected into the pancreatic duct and used per the manufacturer's instructions. Islets were then purified in a gradient solution of Optiprep (Nycomed, Olso, Norway) (25). Human islets were sampled and assessed by dithizone staining (26), pelleted, rinsed with cold PBS, and stored at −80°C. Rat islets were placed in Hank's solution and handpicked before being pelleted, rinsed, and frozen at −80°C. Islet pellets were lysed in Novex sample buffer (Invitrogen) with freshly added protease inhibitor cocktail (P8340; Sigma) and boiled for 4 min. To help verify quality, in preliminary experiments, extracts were immunoblotted for the brain- and islet-specific protein GAD65. The bands formed by the brain extracts comigrated with the islet bands but differed in curvature because of the properties of the buffers in which the extracts were supplied.

Immunoblotting. Samples (15 μg/lane) were run out under reducing conditions on Novex *bis-tris* 10% or 4–12% gels (Invitrogen). Proteins were transferred to polyvinylidene fluoride (PVDF) membrane using NuPage transfer buffer (Invitrogen). Using the method of O'Farrell (27), two-dimensional electrophoresis of human islet protein extract (190 μg/gel) and of ^{35}S-labeled hVIAAT protein and subsequent Western transfer was performed by Kendrick Laboratories (Madison, WI). The Columbia University Howard Huges Medical Institute (HHMI) Protein Chemistry Core (New York) performed mass spectrometer (ms/ms) sequencing of protein spots from a gel run in parallel. Membrane blocking and antibody incubations were done in PBS with 5% nonfat dried milk and 0.05% Tween-20. Antibody dilutions were as follows: NH_2-terminal VIAAT antibody, 1:4,000; COOH-terminal VIAAT antibody, 0.5 μg/ml; anti-glyceraldehyde-3-phosphate dehydrogenase, 0.5 μg/ml; and GC-3108, 1:8,000. Peptide competition was carried out with the COOH-terminal VIAAT peptide or an irrelevant peptide (control), as described for immunostaining. Protein bands were visualized with horseradish-peroxidase–coupled secondary antibody (Chemicon) and enhanced chemiluminescent reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

RESULTS

Sequence of human VIAAT. Human chromosome 20 contains sequences homologous to rodent VIAAT. Three possible exons are situated in the region encoding the putative human VIAAT gene: exon 1 is 390 bp, exon 2 is 957 bp downstream from exon 1 and 231 bp in length, and exon 3, 1,888 bp, is 1,151 bp downstream of exon 2. Exons 1 and 3 are highly homologous to rodent VIAAT. Primers were designed using the genomic data. Amplification of cDNA from human brain yielded a single product consistent with the length of a transcript containing exons 1 and 3; there was no evidence of alternative splicing. The sequence of the PCR product confirmed that human VIAAT is encoded by two exons on chromosome 20. The cDNA sequence was submitted to GenBank (accession AY044836).

hVIAAT, like rat, is a protein of 525 amino acids (Fig. 1). Rat and hVIAAT differ by seven amino acids, with six of the substitutions occurring NH_2-terminal to residue 115. In contrast, the mouse and rat proteins differ by only one residue before amino acid 514. Past residue 514, the mouse sequence lacks homology to the COOH-terminal 11 residues of the human and rat proteins. Computer analysis predicts that hVIAAT, like rodent VIAAT, has 10 transmembrane domains and that, within the cytoplasmic domains, there are nine conserved possible serine phosphorylation sites, including five within the COOH- and NH_2-terminal domains (residues 15, 17, 20, 25, and 511; Fig. 1).

Detection of VIAAT by the COOH-terminal antibody. Immunoblot analysis was used to test for the presence of VIAAT in rat and human islets. The COOH-termini of rat and human VIAAT are identical, so we probed blots of islet and brain tissue extract with an antibody to this region (Fig. 2). As expected, the ~57-kDa VIAAT band was detected in the lane with rat brain extract. Also, a faster-migrating ~52-kDa VIAAT band described in two earlier reports was observed (20,21). Both VIAAT bands were also detected in rat islet extract. In contrast to rat brain, in islet, the ~52-kDa form was the most abundant. In human brain extract, only the ~52-kDa band was detected. Consistent with past results (12), VIAAT was not detected in other tissues, including kidney (not shown), liver, and ovary. Competition with the immunizing VIAAT COOH-terminal

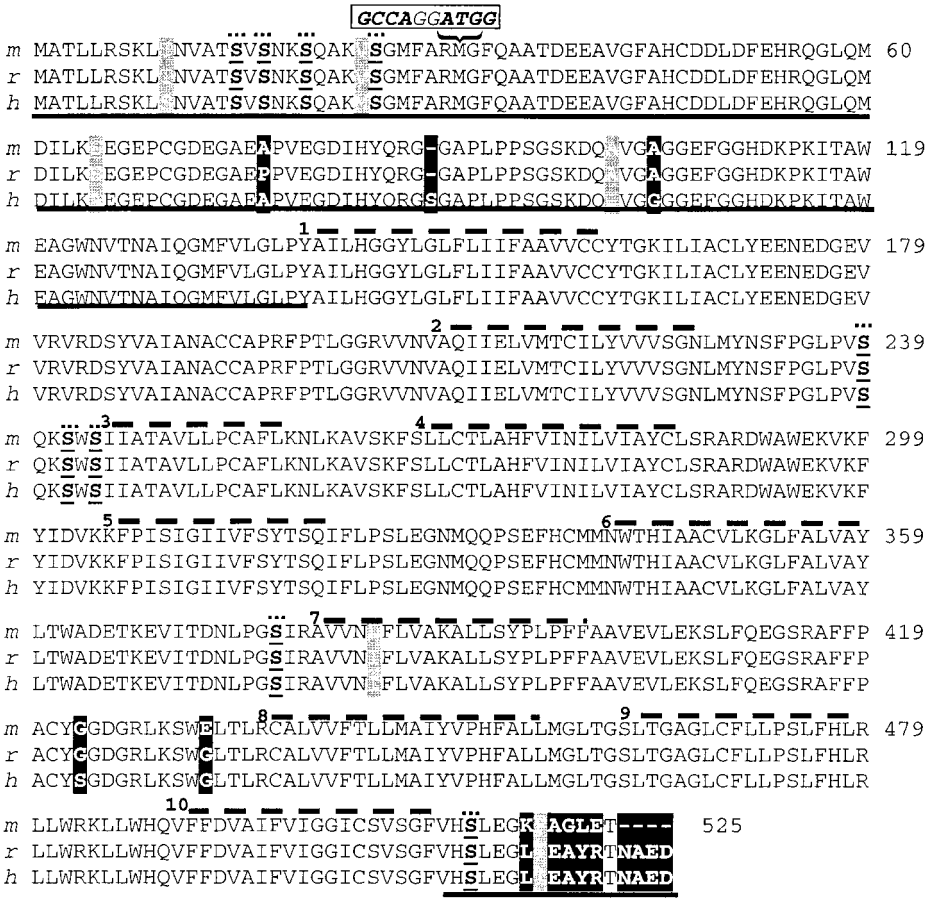

FIG. 1. Alignment of mouse (m), rat (r), and human (h) VIAAT amino acid sequences. Amino acid residues that differ among the three species are shaded: conservative substitutions are shaded gray and substitutions by dissimilar amino acids are shaded black. The predicted cytoplasmic NH_2-terminal and COOH-terminal domains are underlined. Dashes have been placed over the 10 predicted transmembrane segments, which are also numbered. Cytoplasmic serine residues that are likely sites of phosphorylation are indicated (bold "S" underneath dots). The cDNA sequence surrounding the ATG codon encoding methionine residue 31 (shown in box above amino acid sequence) is conserved among the three species. It is a potential alternate start site of translation, with 8 of 10 matches (bold letters) to the vertebrate consensus sequence for translation initiation (34).

peptide prevented detection of the bands, indicating that binding was specific (Fig. 2). In contrast, an ~67-kDa human islet band detected by the COOH-terminal antibody was not efficiently competed. VIAAT was not detected in any of the five human islet extracts tested (two shown).

In vitro expression of VIAAT. Transcription and translation of the hVIAAT cDNA in a rabbit reticulocyte lysate system resulted in the synthesis of both the 57- and 52-kDa forms of the protein (Fig. 3). Addition of microsomal membranes to the reticulocyte lysate enabled membrane-dependent post-translational processing such as signal peptide cleavage (data not shown) and core glycosylation. Without the addition of exogenous membranes, these modifications did not occur (e.g., compare *lanes 3* and *4* in Fig. 3*A*). Both VIAAT forms, however, were synthesized with or without the addition of membranes to the reaction, indicating that formation of a second VIAAT band was not a result of processing by membrane-associated proteins. Also, both bands persisted after treatment of the expressed hVIAAT with calf intestinal phosphatase, as described previously (28) (not shown). Both the 57- and 52-kDa proteins were precipitated by the COOH-terminal antibody and, with greater efficiency, by an antibody (20) raised against the NH_2-terminal 127 residues of VIAAT (the NH_2-terminal antibody; Fig. 3*B* and data not shown).

Detection of VIAAT in islets with the NH_2-terminal antibody. Fig. 4*A* shows that both the 57- and 52-kDa forms of VIAAT are also detected by the NH_2-terminal antibody. The two hVIAAT bands comigrate with the corresponding rat brain bands. A third higher–molecular weight brain band was also observed that likely represents the phosphorylated form of the protein previously detected with the NH_2-terminal, but not the COOH-terminal, antibody (14,28). The 52- and 57-kDa VIAAT bands were also detected in rat islets, confirming that rat islets contain VIAAT (Fig. 4*B*). As observed with the COOH-terminal antibody, brain and islet differ in that, in islet, the 52-kDa form is more abundant. Also, the higher–molecular weight, possibly phosphorylated form is absent in rat islets (Fig. 4*B*).

The NH_2-terminal antibody did not detect VIAAT in human islets by immunoblotting (Fig. 4*B*) or by immunohistochemical staining (not shown). Long exposures of immunoblots loaded with twofold more human, but not rat, islet protein resulted in detection of a weak ~55-kDa band by the NH_2-terminal but not the COOH-terminal antibody. Two-dimensional gel electrophoresis, immunoblotting, and subsequent microsequencing showed that this 255 kDa band did not comigrate with in vitro synthesized hVIAAT in either dimension (not shown) and that it

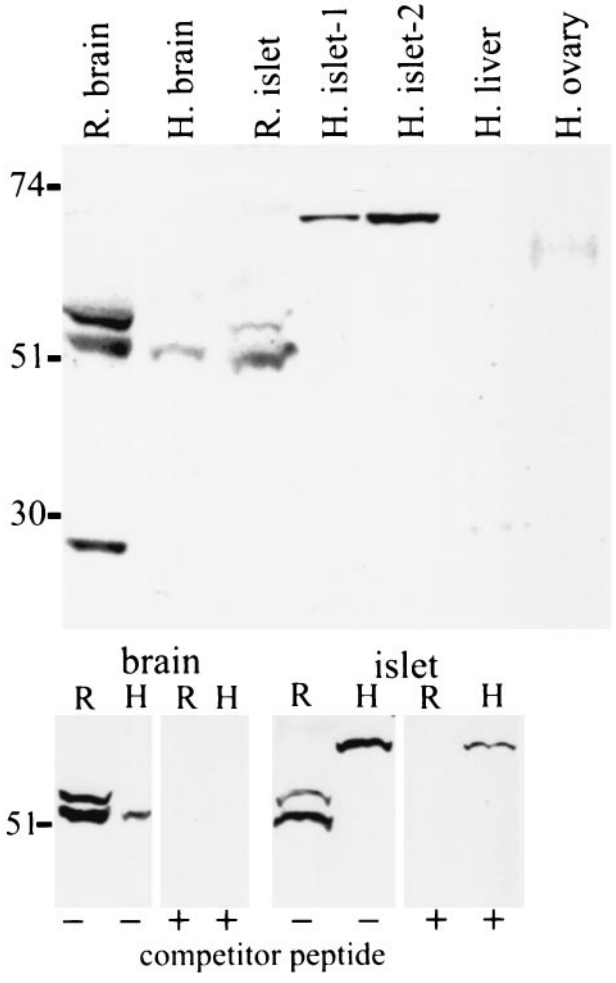

FIG. 2. Immunoblot analysis of VIAAT expression using an antibody to the COOH-terminus. Top panel: VIAAT from rat (R) brain migrates as two bands, with a predominance of the upper ~57-kDa band. Human (H) brain extract contains the ~52-kDa faster-migrating form. Rat islets express VIAAT, with a predominance of the ~52-kDa band. VIAAT was not detected in human islet extracts (data from two different islet preparations shown). The VIAAT band detected in human brain was also not detected in liver and ovary. The identity of the ~67-kDa human islet band is unknown; unlike the VIAAT bands, it was not consistently reproducible. The nearly 30-kDa rat brain band has been observed previously using the same antibody (14); it is not present in rat islet extracts. Bottom panels: The immunizing peptide (competitor peptide) competes with blotted VIAAT for the COOH-terminal antibody's antigen binding site. The competitor peptide (+), but not an unrelated control peptide (−), prevented detection of the two VIAAT bands, which provides evidence of binding specificity.

represented keratin 8, a component of pancreatic acinar, but not islet, tissue (29). VIAAT was not detected. In contrast, the islet-specific protein GAD65 was detected in all human islet extracts tested.

Distribution of VIAAT in rat islets. We used the COOH- and NH_2-terminal antibodies to determine the distribution of VIAAT in rat islets. Immunostaining revealed that VIAAT is most abundant in the mantle (peripheral) region, where glucagon-secreting α-cells and other non–β-cells are located (Fig. 5). Immunostaining with a third antibody, VGAT/1 (21), confirmed this result (not shown). VIAAT is also present in the central β-cell region. The distribution of VIAAT that we observed paralleled that of GAD67. In agreement with a previous study, GAD67 was present in β-cells but was more abundant in the mantle region, whereas GAD65 was β-cell specific (22).

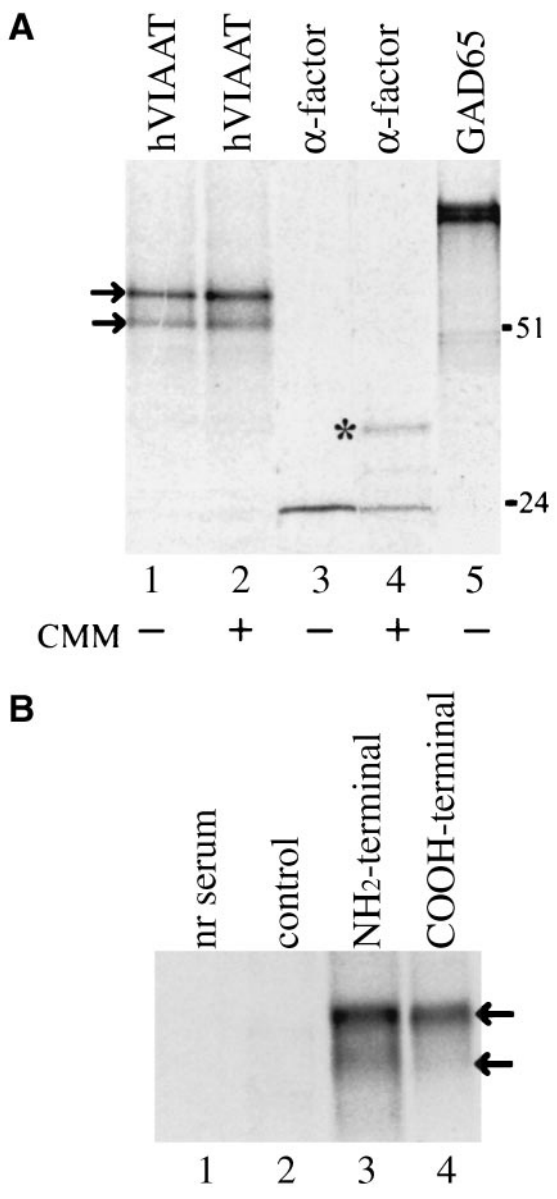

FIG. 3. Gel electrophoresis of radiolabeled proteins from in vitro transcription and translation reactions. *A*: *Lanes 1* and *2*: in vitro synthesis of hVIAAT yielded both the ~52-kDa and ~57-kDa VIAAT bands (arrows). Both forms of VIAAT were produced whether (+) or not (−) canine pancreatic microsomal membranes (CMM) were added to the reaction. *Lanes 3* and *4*: Synthesis of *S. cerevisiae* α-factor. The glycosylated form (asterisk, *lane 4*) was only produced in reactions to which CMM were added. The reticulocyte lysate alone could not mediate membrane-dependent post-translational modifications, indicating that such modifications were not the reason that two VIAAT bands were formed instead of one. *Lane 5*: Reaction using GAD65 cDNA: formation of the 52- and 57-kDa bands was dependent on the addition of hVIAAT cDNA template to the reaction. *B*: The two VIAAT bands (arrows) are precipitated by the NH_2-terminal (*lane 3*) and COOH-terminal (*lane 4*) antibodies. A longer exposure of the autoradiograph to better see the ~52-kDa band confirmed the presence of both VIAAT bands in *lanes 3* and *4*. The VIAAT bands did not precipitate nonspecifically in control immunoprecipitations with normal rabbit serum (nr serum; *lane 1*) or an unrelated (control) polyclonal antibody (*lane 2*).

To confirm the presence of both VIAAT and GAD67 in non–β-islet cells, we immunostained islets from diabetic BB rats (23). DP BB rats develop autoimmune diabetes (30). After the onset of glycosuria, they rapidly lose all of their β-cells, resulting in shrunken islets comprised mostly

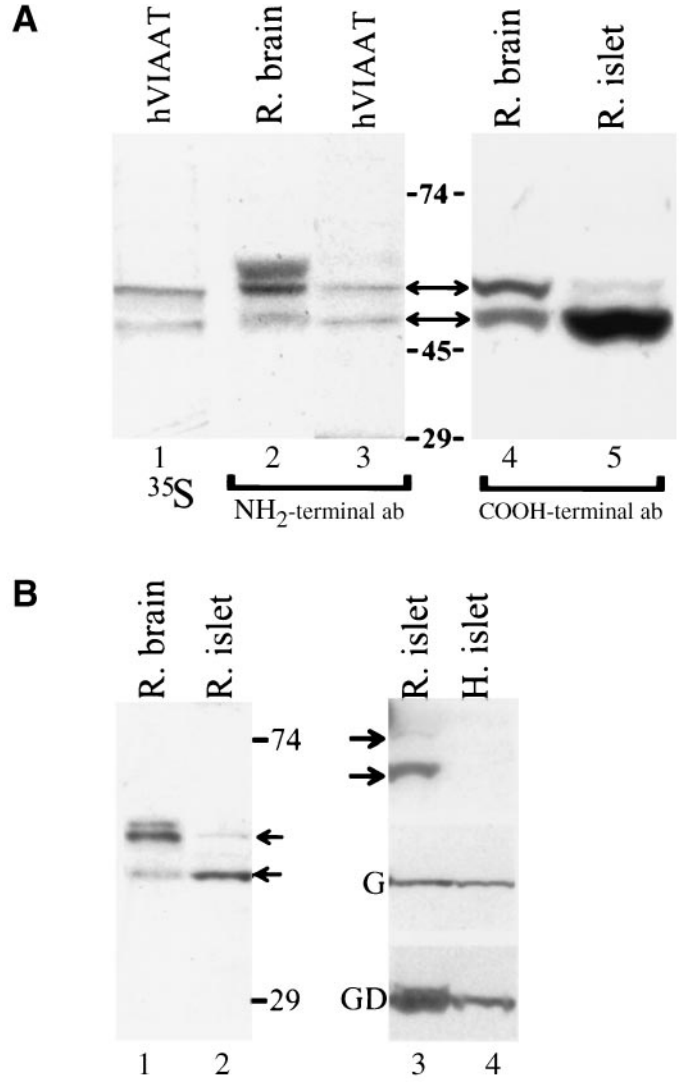

FIG. 4. Detection of VIAAT with the NH_2-terminal antibody. *A*: The NH_2-terminal antibody detects both VIAAT bands in rat brain. *Lanes 2* and *3*: Immunoblot analysis with the NH_2-terminal antibody of rat brain extract (R. brain; *lane 2*) and of in vitro synthesized hVIAAT (*lane 3*). The NH_2-terminal antibody detected both VIAAT bands (arrows). *Lane 1*: In vitro–synthesized radiolabeled hVIAAT (^{35}S) was transferred to the same membrane used for *lanes 2* and *3* and detected by autoradiography. The bands detected by immunoblotting comigrated with the radiolabeled VIAAT. The upper ~57-kDa rat brain band (*lane 2*) was previously shown (in experiments using the same antibody used here) to migrate as a doublet due to phosphorylation (28). The upper (phosphorylated) band was not observed in the in vitro translation product (hVIAAT; *lane 3*). *Lanes 4* and *5*: Rat brain (R. brain) and rat islet (R. islet) proteins were separated using an identical gel and subjected to immunoblot analysis using the COOH-terminal VIAAT antibody (as indicated below the lanes). Protein standards run alongside the extracts were used to align this immunoblot with the blot shown in *lanes 1–3* (NH_2-terminal). The migration of the bands detected by the COOH-terminal and NH_2-terminal antibodies was identical, although the COOH-terminal antibody did not detect the upper (phosphorylated) band. *B*: Immunoblot analysis with the NH_2-terminal antibody of rat brain extract (R. brain; *lane 1*), rat islet (R. islet; *lanes 2* and *3*), and human islet (H. islet; *lane 4*) shows that rat islets, but not human islets, contain detectable amounts of VIAAT (arrows). *Lanes 1* and *2*: Rat islets contain the ~52-kDa and ~57-kDa forms of VIAAT but not the more slowly migrating band present in brain. *Lanes 3* and *4*: Rat islet (*lane 3*) and human islet (*lane 4*) extracts were analyzed in parallel by immunoblotting with the NH_2-terminal antibody. VIAAT is not detectable in the human islet lane. The ~67-kDa protein seen with the COOH-terminal antibody also was not observed. Separate blots of the extracts were probed with monoclonal antibodies to glyceraldehyde-3-phosphate dehydrogenase (G) and to GAD65 and GAD67 (GD). As expected, GAD65 was detected in both extracts, and GAD67 (migrates just above and not well separated from GAD65) was detected only in the rat islet extract (*lane 3*).

of α-cells with relatively reduced numbers of δ- and PP-cells (31,32). Accordingly, the β-cell–depleted islets studied here stained positive for glucagon but not for GAD65 (Fig. 6) or insulin (Fig. 7). Consistent with expression of both GAD67 and VIAAT in rat islet mantle (non-β) cells, the diabetic islets stained positive for both proteins. The presence of GAD67 and VIAAT in the rat islet mantle predicted that GABA should be found there too. In agreement with a prior report indicating that GABA is present in the rat islet mantle, although at lower levels than in the β-cells (33), diabetic islets stained positive for GABA (Fig. 7).

DISCUSSION

The existence of an islet cell vesicular GABA transporter is suggested by the presence of GABA within the SLMV, where it is probably stored before exocytosis despite the cytoplasmic localization of GAD (4). We have found that VIAAT, a transmembrane GABA transporter associated with synaptic vesicles in the brain, is expressed in rat islets.

We determined that human VIAAT is 525 amino acid residues in length and shares nearly 99% sequence identity with the rat protein. There was no evidence of splice variants by PCR, despite suggestive genomic sequence data. VIAAT consists of 10 transmembrane domains with a large NH_2-terminal cytoplasmic domain (~125 residues) and a smaller COOH-terminal cytoplasmic domain (~14 residues) (12,13). Six of the seven residues that differ between rat and human VIAAT lie in the NH_2-terminal cytoplasmic domain. The COOH-terminal domain of the mouse protein diverges from that of the human and rat proteins. The central transmembrane region then is the most highly conserved. In vitro translation of hVIAAT yields two protein bands that comigrate with the two rat VIAAT forms that we observed in rat islet and brain. Surprisingly, we were unable to detect VIAAT in human islets.

Our results indicate that there are at least two forms of the VIAAT protein; these migrate at ~57 and ~52 kDa. Both forms were detected by both an antibody to the COOH-terminus of VIAAT and an antibody raised against the NH_2-terminal 127 residues, and both were formed after in vitro synthesis of VIAAT. The NH_2-terminal antibody also detects an ~59-kDa band in rat brain that was previously shown, using the same antibody, to be due to serine/threonine phosphorylation of VIAAT (28). The failure of the COOH-terminal antibody to detect this band suggests that one site of phosphorylation is the COOH-terminus. Residue 511, a serine, is a predicted phosphorylation site and is part of the peptide sequence against which the COOH-terminal antibody was raised; phosphorylation here may prevent antibody recognition. The ~59-kDa band was not detectable in rat islets.

The pattern of expression in rat islets and brain also differed in that the 57-kDa band was more prevalent in the latter, whereas, in islets, the 52-kDa form was more abundant. The lower abundance in neural tissue may explain why the 52-kDa form is described in only one (21) of several published reports characterizing VIAAT in rodent brain. Transfected COS-7 cells were also found to synthesize the lower–molecular weight molecule (20). It is unclear how the two different forms of the protein differ.

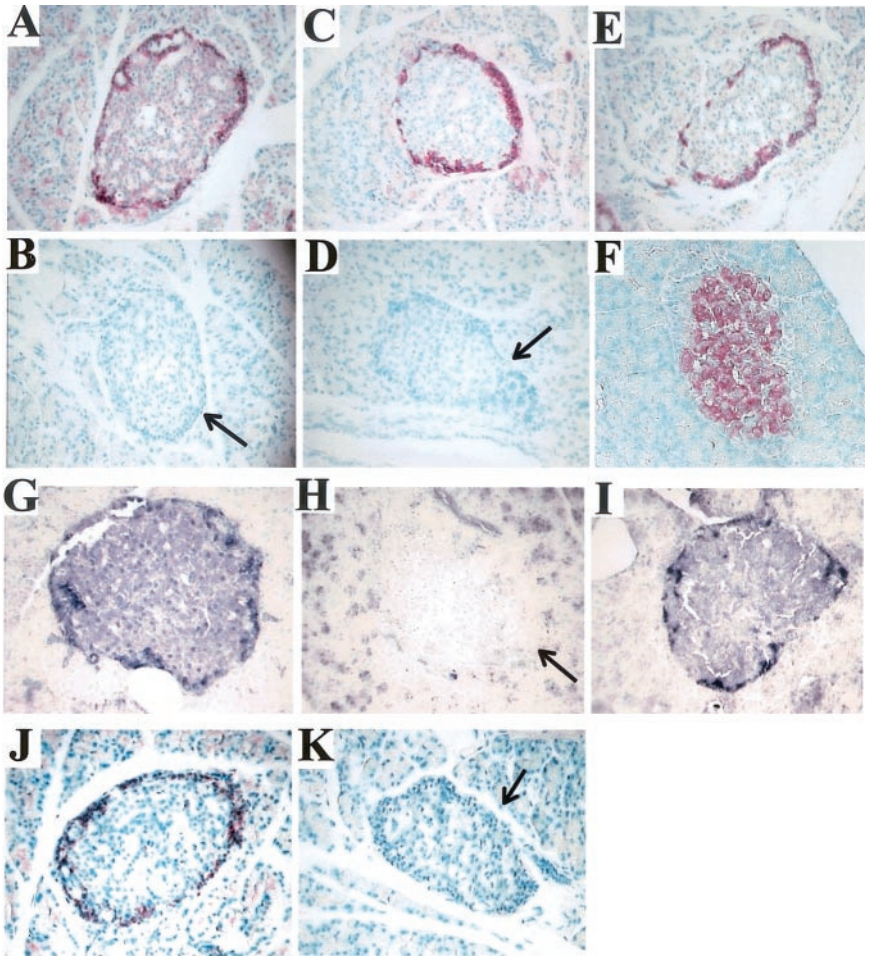

FIG. 5. VIAAT and GAD expression in rat islets: immunostaining of BB rat (nondiabetic) pancreas sections. *A*, *G*, *E*, and *I*: The COOH-terminal antibody detects VIAAT predominantly in the islet mantle (*A* and *G*), similar to the distribution of GAD67 (*E* and *I*). The blue histochemical substrate better shows the less intense β-cell (central) VIAAT (*G*) and GAD67 (*I*) staining. *C*: As expected, glucagon staining localizes to the islet mantle. *B*: Blocking the COOH-terminal antibody's antigen binding site with the immunizing VIAAT peptide prevents islet staining. *D* and *H*: Incubation in parallel with normal rabbit serum does not result in islet staining. *F*: GAD65, as expected, is present in the central β-cell region of the islet. *J* and *K*: The NH_2-terminal antibody, which yielded weaker staining, confirmed that VIAAT was more abundant in the islet periphery, but the staining intensity was not sufficient to detect expression of the protein in the central β-cell region (*J*). Normal rabbit serum used in parallel (*K*) did not result in islet staining. The arrows point to unstained islets. A third antibody to VIAAT, VGAT/1 (21), yielded results identical to those shown in *J*.

In vitro, both forms were synthesized regardless of whether microsomal membranes were added to the reaction. Thus, they are not formed by a post-translational modification, such as core glycosylation, mediated by microsome-bound enzymes. It is also unlikely that the 52-kDa form results from proteolytic cleavage of the larger form because the smaller protein was consistently present in cell extracts prepared in the presence of protease inhibitors, extended incubations of cell extracts or in vitro translation reactions did not result in conversion of the longer form to the smaller, and there was no evidence of similar proteolysis in other proteins studied in parallel. One possibility is that the smaller form results from translation initiation at an alternate start site, such as perhaps the AUG codon encoding residue 31, which, in rat and human, is part (positions +1 to +3) of a stretch of 10 residues (−6 to +4) containing eight matches to the Kozak consensus sequence, including matches at the important –3 (A) and +4 (G) positions (34). Examples of alternate translational start site utilization in mammalian cells have been described (35–37).

The distribution of VIAAT in rat islets parallels that of GAD67. Both were most abundant in the islet mantle, where glucagon-producing α-cells and other non–β-cells are localized. VIAAT was also present in the β-cells but in a lower abundance. Immunostaining of diabetic rat tissue confirmed that both VIAAT and GAD67 were present in non–β-cells. Previous work has demonstrated that, in endocrine cells, as in neurons, VIAAT localizes to microvesicles: in transfected PC-12 cells, VIAAT associated with the endogenous SLMVs (12).

The distribution of GAD67 in rat islets observed in this study matches that described in an earlier report (22). Others, however, have reported that GAD67 is only expressed in rat islet β-cells (38,39). These latter studies relied on in situ nucleic acid hybridization, which did not directly assess GAD protein expression and carried the risk of cross-hybridization with the homologous GAD65 message, on immunoblot analysis using an antibody that recognizes both GAD isoforms and on immunohistochemistry using the same antibody. Rat islets contain significantly more GAD65 than GAD67 (40). As a result, GAD67 may be difficult to detect and distinguish from GAD65 in experiments performed with an antibody that recognizes

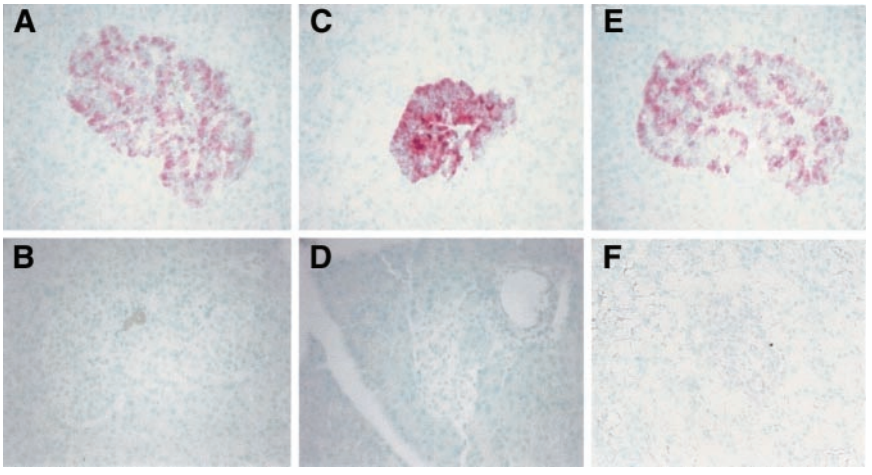

FIG. 6. Immunostaining of VIAAT and GAD67 in pancreas from diabetic rats. *A*: VIAAT is present in the β-cell–depleted islets. The COOH-terminal antibody was used for detection. *B*: Preabsorbing the antibody with the immunizing peptide prevents islet staining. *C* and *F*: Staining for glucagon and GAD65. The diabetic islets contain α-cells, as indicated by glucagon staining (*C*), but are depleted of β-cells, as indicated by the absence of GAD65-containing (*F*) and insulin-containing (Fig. 7*B*) cells. *D*: Incubation with normal rabbit serum did not result in islet staining. *E*: The diabetic islets stained positive for GAD67. *A*, *B*, and *E* show stained sections through the same islet. *C*, *D*, and *F* are different islets from the same pancreas.

both isoforms. Another report described GAD67 expression in glucagon-containing α-cells in rat islet monolayers, consistent with the results presented here (39). GAD65 expression was β-cell specific, which is also in agreement with our data. Besides methodological differences, the conflicting results regarding rat islet GAD67 distribution could be due to variations in GAD expression in different rat strains. This study used BB rat pancreata; other studies used Wistar rats (38,39). Similarly, there have been conflicting reports as to the presence of GABA in rat islet mantle cells. Whereas some studies conclude that GABA is absent (41,42), other reports demonstrate that the islet mantle and also α-cells in rat islet cell monolayers contain GABA, although at lower levels than β-cells (33,39). Here, using islets depleted of β-cells, we confirm that rat islet non–β-cells are capable of synthesizing GABA. This result is consistent with our finding that these cells contain GAD67. Whether insulitis and diabetes altered cellular levels of GABA is unknown.

We were able to compete away COOH-terminal antibody-mediated immunostaining with the immunizing peptide. Also, control stainings with normal rabbit serum and other unrelated antibodies did not result in positive islet staining. It is therefore highly unlikely that our immunostaining results are attributable to nonspecific binding. Also, β-cell–depleted diabetic islets were, as expected, not bound by GAD65 or insulin antisera—further evidence that the islet staining shown herein was specific.

This study is the first to examine VIAAT expression in cells that express only GAD65 or GAD67. In brain tissue, both isoforms are uniformly expressed, with GAD65 constituting from 61 to 89% of GAD content (43). Rat islet β-cells also contain both GAD isoforms. In contrast, human islets express only GAD65, and rat islet mantle cells, as confirmed here, contain just GAD67 (4,22). Whereas GAD65 is mostly bound to the surface of the SLMV, GAD67 is primarily nonmembrane associated, so its role in producing GABA for the SLMV is less clear. Limited trafficking of GAD67 to microvesicles, however, suggests that GAD67 may also produce GABA for SLMV uptake and secretion (8). The expression of VIAAT by rat islet mantle cells is evidence of such a role for GAD67.

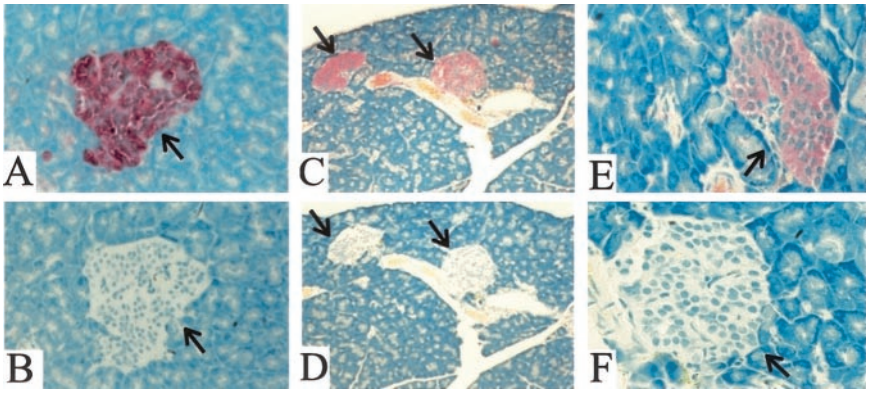

FIG. 7. Immunostaining of diabetic β-cell–depleted rat islets (arrows) reveals that non–β-cells contain GABA. *A* and *B*: Sections from the same diabetic pancreas were stained for glucagon (*A*) and insulin (*B*). As documented previously (31,32), islets in diabetic BB rats are rapidly depleted of virtually all of their β-cells; thus, they do not stain for insulin (*B*) or GAD65 (Fig. 6*F*). *A* and *B* show sections through the same representative islet. *C* and *E*: Diabetic islets were stained with a monoclonal antibody to GABA (*E* is a higher-power view). *D*: Staining of a section through the same islets with a control antibody (to corticotrophin releasing factor; *F*, higher-power view). Tissue sections were photographed at ×400 (*A*, *B*, *E*, and *F*) and ×100 (*C* and *D*).

The parallel distributions of VIAAT and GAD67 in rat islets is striking, especially since GAD65 is the most abundant isoform in the rat islet (40). This pattern of distribution could be due to the relative degrees of association with the SLMV of GAD65 and GAD67. Because GAD65 is mostly bound to the SLMV—and is perhaps in direct contact with VIAAT—GAD65 is probably more efficient in delivering GABA to the microvesicle than GAD67. Perhaps, then, to compensate for this and allow for adequate GABA uptake, VIAAT content in the SLMV membrane is increased in cells that use GAD67 to produce some or all of their GABA stores. Because VIAAT was not detected in human islets, cells that express solely GAD65 may require little VIAAT. Alternatively, a different protein—perhaps the same one that binds GAD65 to the surface of the SLMV—mediates the vesicular uptake of GABA produced by GAD65, and cells with only GAD65 contain no VIAAT.

VIAAT is also a transporter for glycine, which is, like GABA, an inhibitory neurotransmitter (13,14). Further work is necessary to determine whether the SLMVs of islet cells store and secrete glycine.

In summary, we have shown that VIAAT is expressed in rat islets and thus likely mediates the proton gradient–driven vesicular GABA transport activity previously described in rodent islets (10). VIAAT could not be detected in human islet β-cells. Our data confirm a previous report that rat islet mantle cells express GAD67 and do so at greater levels than β-cells (22). We have also confirmed that rat islet non–β-cells can produce GABA. VIAAT expression in the rat islet, which is greatest in the islet periphery, parallels the distribution of GAD67 expression. We hypothesize that this is because the localization of GAD65 to the SLMV membrane allows efficient GABA transport that requires little or no VIAAT, whereas cells that contain GAD67 require relatively increased levels of VIAAT expression to facilitate GABA entry into the SLMV. The GAD-GABA-VIAAT signaling machinery in islets exhibits great heterogeneity, ranging from human β-cells, which express high levels of GAD65, no GAD67, and undetectable levels of VIAAT, to rat mantle cells, which contain only GAD67, relatively low levels of GABA, and increased levels of VIAAT. Further work will be necessary to characterize how the 57- and 52-kDa forms of VIAAT differ in structure and function to determine why there are relatively increased levels of the smaller form in rat islets and to determine whether they interact differently with GAD65 and GAD67.

ACKNOWLEDGMENTS

This research was funded by grants to S.D.C. from the Juvenile Diabetes Research Foundation International (JDRF) and the National Institutes of Health (K08-DK02944) and by grant DK26190 to Åke Lernmark. The Human Islet Transplantation in Seattle islet distribution program was funded by JDRF.

We thank Angela Wallen for technical assistance with islet isolation. Dr. Bruno Gasnier graciously provided an antibody to VIAAT. Rodent islet isolations were performed by the Islet Satellite of the University of Washington DERC Cell and Tissue Core (DK17047). The DERC Cytohistochemistry Core performed some of the immunohistochemical staining shown herein; we thank Joyce Murphy for her help in this regard. The DERC Molecular Genetics Core assisted with cDNA cloning and sequencing, for which we thank Dr. Libby Rutledge, Brian Van Yserloo, and Paul Gohlke. We thank Åke Lernmark for his helpful comments regarding this manuscript, and we also thank Bunny Williams for her kind support.

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