Identification and characterization of a novel isoform of the vesicular γ -aminobutyric acid transporter with glucose-regulated expression in rat islets

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Abstract

Pancreatic islets are unique outside the nervous system in that they contain high levels of the inhibitory neurotransmitter γ -aminobutyric acid (GABA), synthesized by the enzyme glutamic acid decarboxylase (GAD). Since the role that GABA plays in the islet and the mechanisms whereby the two major GAD isoforms (GAD65 and GAD67) function as diabetes-associated autoantigens are unknown, continued characterization of the islet GAD–GABA system is important. We previously demonstrated that the GABA and glycine transporter vesicular inhibitory amino acid transporter (VIAAT also known as VGAT) is present in rat islets. Here we identify a novel 52 kDa variant of VIAAT in rat islets: VIAAT-52 (V52). V52 is an amino-terminally truncated form of VIAAT (V57) that likely results from utilization of a downstream start site of translation. V57 and V52 display different patterns of post-translational modification and cellular expression. Our results have indicated that islet content of V52, but not V57, is responsive to changes in glucose concentration and other extracellular conditions. VIAAT is expressed in the islet α cells, but there have been conflicting findings regarding the presence of VIAAT in the β cells. Here we have also provided additional evidence for the presence of VIAAT in islet β cells and show that the β cell line INS-1 expresses V57. V52 may be better adapted than V57 to the unique rat α cell GAD–GABA system, which lacks GAD65 and in which VIAAT traffics to secretory granules rather than just to synaptic microvesicles.

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Introduction

A distinctive feature of pancreatic islets is that they synthesize and store γ -aminobutyric acid (GABA). Despite much work, though, the role of GABA in the endocrine pancreas remains unknown (Satin & Kinard 1998, Chessler & Lernmark 2000). In the central nervous system, GABA is the major inhibitory neurotransmitter. In the islets, it probably also functions as an intercellular signaling molecule, perhaps – as there is evidence to suggest – as a paracrine or autocrine regulator of insulin or glucagon secretion (Chessler & Lernmark 2000, Braun *et al.* 2004a,b). Recent evidence suggests that a second inhibitory neurotransmitter, glycine, also plays a role in islet function (Gammelsaeter *et al.* 2004).

Characterization of the cellular GABA signaling machinery in islets will likely yield important insights into the role of GABA in islet function. The GABA system is also of great interest because the enzymes that catalyze the synthesis of GABA – glutamic acid

decarboxylase (GAD)65 and GAD67, the two major isoforms of GAD – are key autoantigens associated with human (in the case of GAD65) and NOD mouse (in the case of both GAD65 and GAD67) autoimmune diabetes (Elliott *et al.* 1994, Lernmark 1996). The interactions of GAD65 and GAD67 with other protein constituents of the islet GAD–GABA system may help mediate the intracellular trafficking of the two enzymes, their characteristic association with microvesicles, and, perhaps, therefore, their distinctive ability to function as autoantigens (Solimena 1998).

In the central nervous system, the vesicular inhibitory amino acid transporter (VIAAT) is a key component of the GABAergic and glycinergic signaling machinery. VIAAT is a transmembrane protein that utilizes a proton electrochemical gradient to transport GABA and glycine into synaptic vesicles where they are stored prior to release (Gasnier 2004). Due to the importance of characterizing the pancreatic islet GAD–GABA system, we previously investigated the distribution of VIAAT in rat islets. We found that the rat islet mantle (the islet

periphery, where the α cells are found) harbors a unique GAD–GABA signaling apparatus, characterized by the presence of GAD67, GABA, and VIAAT but not GAD65 (Chessler *et al.* 2002). Lower levels of VIAAT were also detected in the rat islet β cells, which contain GAD65 (Chessler *et al.* 2002). Hayashi *et al.* (2003a) subsequently confirmed the presence of VIAAT in the rat islet mantle, detecting none in the islet β cells. Unexpectedly, VIAAT was found to localize to the glucagon-containing secretory granules rather than to the synaptic-like microvesicles also present in islet α cells. In contrast, Gammelsaeter *et al.* (2004) identified VIAAT in both secretory granules and microvesicles in rat islet α cells and also detected comparatively low levels in the β cells.

VIAAT is a 57.4 kDa protein (V57). A phosphorylated form (V57') that migrates more slowly by SDS-PAGE is observed in brain tissue extracts (Bedet *et al.* 2000). We found that islet extracts, in contrast to neural tissue, contain little or no V57' (Chessler *et al.* 2002). Unexpectedly, immunoblotting also revealed a second, more rapidly migrating form of the protein in islet and brain extracts. This form was predominant in islet cell extracts and has an apparent molecular weight of 52.5 kDa (V52). Hayashi *et al.* (2003a), using a novel antibody that may have a relatively lower affinity for V57, observed only an ~52 kDa VIAAT band in rat islet extracts and α TC6 cells, while both V57 and V52 were detected in brain. The ~52 kDa form of VIAAT is present in low abundance in rat and human brain tissue extracts and is detected, as well, in purified synaptic vesicles and rat pineal gland extracts (Takamori *et al.* 2000, Chessler *et al.* 2002, Jin *et al.* 2003, Echigo & Moriyama 2004). COS-7 and tsA201 cells transfected with different VIAAT cDNA constructs also express an ~52 kDa form of the protein along with the more abundant 57 kDa form (Dumoulin *et al.* 1999, Takamori *et al.* 2000). Using rat and human brain extracts, we found that the 52.5 kDa VIAAT proteins from neural and islet cells co-migrate by SDS-PAGE and are therefore most likely identical (Chessler *et al.* 2002). Similar data suggest that V52 from islet and brain extracts is identical to that synthesized after VIAAT cDNA is either transfected into tsA201 cells or introduced into reticulocyte lysates for *in vitro* transcription and translation (Takamori *et al.* 2000, Chessler *et al.* 2002).

Due to the relatively low abundance of V52 in neural tissue as compared with islet cells, evidence pointing to the existence of a 52 kDa isoform of VIAAT has been previously overlooked. Here, we have identified V52 as a novel variant of VIAAT truncated at the amino terminal end and differentially modified. We have also shown that V57 is present in INS-1 cells and have used immunohistochemistry to provide further evidence of the presence of VIAAT in islet β cells.

Herein, we have also tested the hypotheses (1) that V52 is formed by utilization of an alternate start site of translation and (2) that its level of expression is independent of that of V57 and responsive to glycemic conditions.

Materials and methods

Antibodies

The antibody referred to as VT/510 is an affinity-purified antibody to the carboxyl terminus of VIAAT (Chemicon, Temecula, CA, USA). VT/75 (Synaptic Systems, Göttingen, Germany) is an affinity-purified antibody to VIAAT residues 75–87. A monoclonal antibody raised against the same peptide (M-VT/75) was also purchased from Synaptic Systems. VT/75 and M-VT/75 are well characterized and have been used extensively to detect VIAAT in neural and testicular tissue, to specifically identify inhibitory synapses and to immunopurify VIAAT-carrying microvesicles (see, for example: Geigerseder *et al.* 2003, Prange *et al.* 2004, Chih *et al.* 2005, Saito *et al.* 2005). VT/17 was made to our specifications by Alpha Diagnostic, Inc. (San Antonio, TX, USA) in rabbit hosts by coupling a peptide representing VIAAT residues 17–30 (CSNKSQAKVS GMFAR) to keyhole limpet hemocyanin. In preliminary immunoblotting experiments, VT/17 was found to recognize the 57 kDa VIAAT band in rat brain and islets but did not detect a 57 kDa protein in three tissues previously shown not to express VIAAT: rat kidney, liver and skeletal muscle (McIntire *et al.* 1997). Preimmune serum from the same rabbit used to generate VT/17 was retained for use as a negative control. VT/17 was not suitable for immunohistochemistry using either frozen or paraffin-embedded tissues. A monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase was from Chemicon and another to the amino terminus of GAD65 was a kind gift from Dr Åke Lernmark (Hampe *et al.* 2001). Rat brain extract was purchased from Stressgen (Victoria, BC, Canada).

In vitro expression of VIAAT

Transcription and translation of VIAAT cDNA to produce radiolabeled VIAAT in rabbit reticulocyte lysates was carried out as described previously (Chessler *et al.* 2002). The Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce point mutations into the VIAAT cDNA. A T>C substitution at cDNA position 2 was used to generate mutation M1T, and M31 L was created by changing A>C at position 91 and G>A at position 93. M1T-6 xHis was made by utilizing PCR to introduce a 6 xHis tag on the 3' end of VIAAT M1T: VIAAT M1T

was amplified with the primers GACCAAGCTTGCC GCCAC (5') and TTAATGATGATGATGATGATG GTCCTCCGCGTTGGTT (3') and the resulting product then cloned into PCRII-Topo (Invitrogen, Carlsbad, CA, USA). Full-length sequencing of all plasmid inserts confirmed that the constructs were as expected. 6 xHis-tagged V52 was purified using His-Select nickel affinity gel (Sigma, St Louis, MO, USA). Purified protein was sent to the Harvard Microchemistry and Proteomics Facility (Cambridge, MA, USA) for N-terminal sequencing after being prepared and blotted onto Sequi-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) as specified by the microsequencing facility.

Islet preparation

Islets were isolated from the pancreata of diabetes-resistant (DR) BioBreeding (BB) rats (Bieg *et al.* 1998) by digestion with Liberase (Roche Molecular Biochemicals, Indianapolis, IN, USA) followed by Optiprep (Nycomed, Oslo, Norway) gradient purification as previously described (Chessler *et al.* 2002). Isolated islets were hand-picked in Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA). They were then either rinsed with phosphate-buffered saline (PBS) and lysed or cultured in RPMI (Invitrogen) with 3, 10 or 20 mmol/l glucose and supplemented with 10% fetal bovine serum. Islets were lysed at 4 °C in Novex NuPage sample buffer (Invitrogen) or SDS boiling buffer (5% SDS, 10% glycerol and 60 mmol/l Tris, pH 6.8; four islets/μl) and the extracts were either boiled for 4 min or heated for 7 min at 70 °C. At the time of islet lysis, detergent buffers were supplemented with a protease inhibitor cocktail (P8340; Sigma) and 1 mmol/l phenylmethylsulfonyl fluoride. To test lysis in non-ionic detergent, islets were incubated for 30 min in a solution of 1% Nonidet P40 (NP-40) in PBS, and the nuclei and other debris pelleted by spinning for 10 min at 10 000 *g*. Islets not immediately lysed were stored frozen at -80 °C.

INS-1 cells

INS-1 cells were a kind gift from Christopher Rhodes (Pacific Northwest Research Institute, Seattle, WA, USA). INS-1 cells from the Rhodes laboratory have been successfully used by that laboratory and others as a model of β cell biology in the publications cited here and others (Dickson *et al.* 2001, Wrede *et al.* 2002, 2003, Lingohr *et al.* 2003). One of us (I R S) has performed perfusion experiments using the INS-1 that we received and that were in the same passage number range as the cells used for the experiments described herein (Sweet *et al.* 2002). These perfusion experiments demonstrated appropriate responses to glucose and epinephrine and as well as sulfonylurea (glibenclamide) binding (Sweet *et al.*

2002). Using an insulin ELISA (Crystal Chem. Inc., Downers Grove, IL, USA), we have periodically evaluated glucose-stimulated insulin secretion using static culture and similarly determined that the cells had maintained glucose responsiveness (data shown herein and other data not shown). Immunohistochemistry reveals uniform insulin expression by this INS-1 line, with all cells expressing insulin (data shown herein and other data not shown).

INS-1 cells were cultured in medium with 11 mM glucose as described (Sweet *et al.* 2004). Cell extracts were prepared in the same manner as islet extracts. Protein content of cell and islet extracts was determined with the DC protein assay (Bio-Rad) to ensure that equal amounts of protein were loaded into gel lanes.

Subcellular fractionation

INS-1 cells were fractionated using the method of Clift-O'Grady *et al.* (1998). Briefly, cells were pelleted at 800 *g* for 5 min, resuspended in budding buffer (38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM 4-morpholinepropanesulfonic acid, pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate and 2.5 mM magnesium sulfate) and pelleted again at 800 *g* for 5 min. Cells were resuspended in budding buffer with protease inhibitors (Roche Molecular Biochemicals) and homogenized using a Balch-designed cell cracker (European Molecular Biology Laboratory, Heidelberg, Germany). Trypan blue exclusion was used to ensure efficient homogenization. The resulting homogenate was sedimented in an SS34 rotor at 1000 *g* for 5 min to obtain a low speed supernatant (S1). S1 supernatants were then sedimented at 27 000 *g* for 35 min to generate a high speed supernatant (S2) and pellet (P2). To verify exclusion of secretory granules from the S2 supernatant, the supernatant was fractionated by velocity gradient centrifugation (Clift-O'Grady *et al.* 1998) and the fractions evaluated for chromogranin A content by western blot analysis. The S2 supernatant utilized for the experiments shown herein contained synaptophysin, a marker of synaptic-like microvesicles and, as expected, lacked chromogranin A, which instead was found in P2 (A T Suckow, unpublished observations). Tissue extract from BB (DR) rat brains and crude synaptic vesicle preparations were made as previously described (Jin *et al.* 2003).

Immunofluorescence

Cells were seeded on four-well chamber slides (BD Biosciences, San Jose, CA, USA) coated with poly-L-lysine and allowed to grow for 48 h. They were fixed in a 4% paraformaldehyde solution for 1 h, washed twice in 1 × PBS and permeabilized and blocked with PBS containing 0.2% Triton-X-100 and 3% bovine serum

albumin (BSA) for 1 h. Cells were then incubated with a 1:200 dilution of mouse anti-insulin and of rabbit VIAAT antibody for 1 h. After incubation, cells were washed three times with PBS and incubated with a 1:200 dilution of AlexaFluor568 anti-mouse IgG conjugate, a 1:200 dilution of AlexaFluor488 anti-rabbit IgG and a 1:1000 dilution of To-pro 3 (Molecular Probes, Eugene, OR, USA). Confocal microscopy was performed on an FV1000 confocal microscope configured with an Argon/Krypton laser (488 and 568 nm excitation lines) (Olympus, Melville, NY, USA).

Insulin secretion assays

Cells were grown on 12-well plates 48 h prior to measuring insulin then washed twice with Krebs–Ringer bicarbonate buffer containing 0.2% BSA and 2.5 mM glucose. They were then incubated for 30 min in this buffer. After incubation, this solution was removed and replaced with Krebs–Ringer containing either 2.5 mM glucose or 25 mM glucose. Cells were incubated for 2 h, the media were removed and insulin content was measured using a rat insulin ELISA kit (Crystal Chem, Inc.).

Immunohistochemistry

Paraffin-embedded rat and human pancreas tissue were deparaffinized and blocked in a 1% BSA/PBS solution for 1 h at room temperature. Sections were incubated with a 1:200 dilution of primary antibody for 1 h. They were washed twice in $1 \times$ PBS for 10 min and incubated 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. Vector Red (Vector Laboratories, Burlingame, CA, USA) was used to visualize the binding of the primary antibodies. Sections were counterstained with hematoxylin (Fisher Scientific, Fairlawn, NJ, USA) using standard methods.

Protein electrophoresis

Proteins were run on NuPage gels (Invitrogen) and immunoblotted as previously described (Chessler *et al.* 2002). For quantitation, immunoblots were incubated with ECL-Plus chemiluminescent substrate (Amersham Biosciences, Piscataway, NJ, USA) and directly analyzed using a Molecular Dynamics Storm 840 imager and ImageQuant software (Amersham). Otherwise, blots were incubated with ECL substrate (Amersham) and the chemiluminescent signal captured by XAR film (Kodak, Rochester, NY, USA).

Two-dimensional (2-D) electrophoresis was performed according to the method of O'Farrell (1975) by Kendrick Labs, Inc. (Madison, WI, USA). To enhance the solubility of membrane proteins, islet samples were

prepared in SDS boiling buffer (Ames & Nikaido 1976). No urea was added to the sample buffer, and samples were kept frozen at -80°C , avoiding any risk of carbamylation (McCarthy *et al.* 2003). Isoelectric focusing was carried out in glass tubes using 2% pH 3.5–10 ampholines (Amersham). After equilibration, the tube gels were sealed to stacking gels on top of 8% acrylamide slab gels for SDS-PAGE. Two 2-D gels were run in parallel: one with 200 μg islet extract, which was analyzed after Western transfer to polyvinylidene fluoride membrane, and another with 10 μl radiolabeled VIAAT spiked into 200 μg islet extract, which was analyzed by autoradiography (using a storage phosphor screen) and phosphorimaging with a Storm 840 imager. A tropomyosin marker was added to each sample, and molecular weight markers (Sigma) were also run in the second dimension. Tropomyosin yields a characteristic pattern of spots which was observed in all 2-D gels run. To allow precise alignment of the resulting images, the western blot and the gel used for autoradiography were both stained with Coomassie blue to allow visualization and alignment of islet and marker proteins. Alignment was performed electronically using scanned images.

Statistical analysis

Data are presented as means $\pm$ S.E. Differences between quantitative data sets were analyzed by one-way ANOVA. $P < 0.05$ was considered significant.

Results

Synthesis and stability of VIAAT

In vitro transcription and translation of VIAAT cDNA yields significant quantities of two proteins: V52 and V57. Parallel reactions with GAD67, GAD65, luciferase and *Saccharomyces cerevisiae* α -factor cDNAs, in contrast, yielded significant amounts only of the expected protein: the appearance of a prominent, lower molecular weight protein was unique to VIAAT (Chessler *et al.* 2002 and data not shown). To verify that V52 does not result from proteolytic degradation, the time-course of V52 and V57 synthesis was analyzed (Fig. 1A). In repeated experiments, we consistently found that V52 and V57 appeared simultaneously: accumulation of V52 was not preceded by accumulation of V57. Furthermore, the amount of V52 relative to V57 did not increase over time, as would be expected if V57 were gradually converted to V52.

We also investigated the relative stability of both proteins in islet cell extracts, both to test for evidence of proteolytic activity that converts V57 to V52 and also to ensure that V52 is not formed as an artefact of islet cell lysis. We lysed isolated islets in both ionic (SDS) and non-ionic (1% NP-40) detergent solutions both with and

Figure 1 Synthesis and stability of V57 and V52. (A) VIAAT cDNA was added to an *in vitro*, reticulocyte lysate expression system containing ^{35}S -methionine (time 0). Aliquots of the reaction mix were withdrawn at the times indicated above the lanes (left panel), and radiolabeled proteins were analyzed by SDS-PAGE and autoradiography. V52 and V57 appeared simultaneously 15 min after the addition of the VIAAT cDNA to the reticulocyte lysate, and there was no increase over time in the ratio of V52:V57. Immunoprecipitation of the reaction mixture after 70 min (right panel) with two different anti-VIAAT antibodies, VT/510 (lane a) and VT/75 (lane b), verified that the bands running at 57 kDa and 52 kDa were isoforms of VIAAT. A control immunoprecipitation was also performed with normal rabbit serum (NS). (B) Islet proteins were extracted in NP-40 lysis buffer without addition of protease inhibitors and incubated at 37 °C for the amount of time indicated. V52 and V57 protein content was analyzed by immunoblotting with antibody VT/510. Both isoforms are stable in the lysis buffer. In experiments such as this with both non-ionic and ionic (SDS) detergent buffers and both with and without protease inhibitors, there was no evidence of conversion of V57 to V52.

without addition of protease inhibitors. Each type of lysis buffer performed equally well in extracting V52 and V57 from purified islets (data not shown). The lysates were incubated at 4 °C or 37 °C and aliquots were withdrawn at set time-points. To determine whether there was an increase with time in the ratio of V52 to V57, which would be consistent with post-lysis conversion of V57 to the smaller isoform, the VIAAT content of the islet extract samples was examined by immunoblotting. One such experiment is depicted in Fig. 1B. A similar approach was used by Bedet *et al.* (2000) to demonstrate that V57' (a phosphorylated form

Figure 2 VIAAT synthesis from mutated cDNA templates. (Lanes 1–3) VIAAT (lane 1), VIAAT M31 L (lane 2) and VIAAT M1T (lane 3) were synthesized in rabbit reticulocyte lysates in the presence of ^{35}S -methionine[$q;8$] and analyzed by SDS-PAGE and autoradiography. As seen in lane 3, mutation of the initiator methionine (M1T) suppressed synthesis of V57 but not V52. (Lanes 4–6) To ensure that an isoform of VIAAT was synthesized from the M1T template despite mutation of the initiator codon, the M1T reaction product was immunoprecipitated with two anti-VIAAT antibodies, VT/510 (lane 4) and VT/75 (lane 5). The 52 kDa protein yielded by the M1T template was recognized by both antibodies. Normal rabbit serum was used as a control for non-specific binding (lane 6).

of V57) is gradually converted to V57 in brain extracts. In contrast, V57 was not converted into V52: there was no evidence of an increase in V52 content at the expense of V57 content during incubation at either 4 °C or 37 °C in either type of detergent lysis buffer. The same result was obtained when radiolabeled, *in vitro*-translated VIAAT was incubated in islet extracts and then analyzed by autoradiography (data not shown).

The VIAAT gene contains an alternate start site of translation

To test whether synthesis of V52 could be due to the utilization of an alternate, downstream start site of translation, we analyzed VIAAT protein expression after mutating the V57 initiator codon (V57-M1T). As shown in Fig. 2, V52 was preferentially translated after mutation of VIAAT codon 1.

The ATG codon encoding methionine residue 31 occurs in a region of high homology to the eukaryotic consensus sequence for translation initiation (Kozak 1999) and is thus a candidate for an alternate translation start site. While alteration of this downstream codon suppressed synthesis of V52, the degree of suppression was, for unclear reasons, inconsistent (Fig. 2 and data not shown). As expected, the M>L substitution at residue 31 did not affect synthesis of V57.

Figure 3 Immunorecognition of V57 and V52 by antibodies to the amino and carboxyl termini of VIAAT. (Lanes 1 and 2) A rat islet extract was immunoblotted with antibodies to the carboxyl terminus (VT/510, lane 1) and to the amino terminus (VT/17, lane 2) of VIAAT. (Lanes 3 and 4) Immunoblots of a rat brain extract using antibody VT/17 (lane 3) and preimmune serum from the same rabbit (PI). VT/17 exhibits specific recognition of V57 but does not bind V52. Detection of V52 by the carboxyl-terminal antibody (VT/510; lane 1, lower band) but not the amino-terminal antibody (VT/17; lanes 2 and 3) shows that V52's lower molecular weight (compared with V57) is attributable to an amino-terminal truncation.

The formation of V52 by utilization of a downstream start site of translation and the recognition of this protein by an antibody to the carboxyl terminus of VIAAT suggests that it is truncated at its amino-terminal end relative to V57. To confirm this, western blots of islet extracts were probed with an antibody to VIAAT residues 17–30 (VT/17). Consistent with the hypothesis that the two VIAAT variants differ at their amino-terminal ends, VT/17 recognized V57 but not V52 (Fig. 3).

To isolate V52, we introduced a 6 xHis tag on the carboxyl terminus of V57-M1T. V52–6 xHis was preferentially translated from this construct and could be captured on nickel agarose beads (not shown). This confirmed that the carboxyl terminus of VIAAT is intact in V52 (since the 6 xHis tag remained functional). Captured V52–6 xHis was sent for amino-terminal sequencing. Sequencing, however, was unsuccessful, perhaps due to N-terminal blockage, a common problem with eukaryotic proteins (Brown & Roberts 1976, Persson *et al.* 1985).

2-D gel analysis of V52 and V57

In order to analyze the pattern of post-translational modification of islet VIAAT and to confirm the identity of *in vitro* synthesized V52, VIAAT was detected by immunoblotting rat islet proteins separated using 2-D non-equilibrium pH gradient electrophoresis (NEPHGE)/SDS-PAGE (O'Farrell 1975). As seen in

Fig. 4, islet V57 runs as a 'train' of five to eight spots. This indicates that post-translational modifications result in multiple forms, but does not indicate the specific nature of the modifications. In contrast, islet V52 appears only as a single spot.

To verify that islet V52 and the reticulocyte lysate-derived V52 synthesized after mutation of codon 1 are the same, we asked whether they would co-migrate during 2-D electrophoresis. Radiolabeled V52 was prepared in the reticulocyte lysate system, spiked into islet extract and loaded onto a 2-D gel. This gel was run in parallel with the gel used to create the immunoblot shown in Fig. 4. The spots yielded by the radiolabeled V52 (Fig. 4Pi) aligned with the immunoblot at the positions indicated (Fig. 4A). As shown in Fig. 4A, the pattern of modification undergone by V52 synthesized in rabbit reticulocyte lysates was similar to that of islet-derived V57, with the protein separating into five forms in the same pI range as V57. We previously observed that bands representing V52 protein derived from islet and brain extracts and from *in vitro* synthesis co-migrate by SDS-PAGE (Chessler *et al.* 2002). In the present study, we found that reticulocyte lysate-derived V52, in the case of the form with the most acidic pI, precisely co-migrates with rat islet V52 during 2-D electrophoresis, suggesting that they are identical (Fig. 4A). Though membrane proteins can be well resolved during NEPHGE/SDS-PAGE, variable protein loss is a common problem and likely explains what, in Fig. 4, appears to be a greater abundance of V57 than V52 (Ames & Nikaido 1976, Chevallet *et al.* 1998, Santoni *et al.* 2000).

The 2-D gel results showed V57 to consist of a heterogeneous group of differentially modified VIAAT molecules (Fig. 4A and B). Some of this heterogeneity was also apparent on standard 1-D gels, with brain-derived V57 frequently seen to migrate as a doublet: V57 and V57' (Bedet *et al.* 2000, Chessler *et al.* 2002). We previously showed that this differential modification results in differential detection of V57 by antibodies, likely due to specific modifications masking targeted epitopes (Chessler *et al.* 2002). VT/510, for example, does not detect human brain V57 or V57' on western blots (Chessler *et al.* 2002). Differential recognition of differently modified forms of V57 is probably the reason why the pattern of V57 spots seen with VT/75 and VT/510 varies (Fig. 4A and B). Differing detection of V57 by different antibodies is also seen in Fig. 4D, with VT/75 preferentially binding more slowly migrating forms in brain extracts (lanes 1 and 2) as compared with VT/510 (lanes 3 and 4).

Differential regulation of V52 and V57 expression

Levels of VIAAT expression in the brain have been reported to be responsive to agonists and antagonists of

Figure 4 2-D electrophoresis of VIAAT. Two NEPHGE/SDS-PAGE gels were run in parallel to analyze proteins from rat islets (by immunoblotting) and radiolabeled V52 synthesized *in vitro* using a VIAAT cDNA construct with a mutated initiator codon. (A) Immunoblot analysis of the rat islet extract using antibody VT/510. V57 separated into a train of five spots (small arrows), indicating that there are at least five differentially modified forms of the protein. V52, in contrast, migrated as one spot (larger, bold arrow). Pi, *in vitro* synthesized, radiolabeled V52 was spiked into islet extract and run in parallel on a separate 2-D gel. It also separated into five differentially modified species. The phosphorimage (Pi) was aligned with the immunoblot (A) as described in Materials and methods to allow direct comparison of the spots generated by *in vitro* synthesis with those derived from the islet extract. One of the five VIAAT species detected by phosphorimaging precisely co-migrated with endogenous islet V52 (indicated by the bold arrow). The other four forms ran where indicated by the circles drawn on the immunoblot. (B) The immunoblotted membrane was stripped and probed with a second anti-VIAAT antibody, VT/75. The spots depicted by the five small arrows and one large arrow co-migrate with the spots shown in (A). VT/75 also detected what are likely three additional V57 species (marked by asterisks) with more acidic pI values. (C) The immunoblotted membrane was stripped and probed with normal rabbit serum. The four spots present in the upper left-hand corner of this panel and (A) and (B) likely represent non-specific antibody binding. (D) Immunoblot of rat brain extract (lanes 1 and 3) and rat brain synaptic vesicle preparation (lanes 2 and 4) with VT/75 (lanes 1 and 2) and VT/510 (lanes 3 and 4). As in (A) and (B), different patterns of recognition of V57 species can be seen depending on which antibody is used. For example, in lane 1, VT/75 can be seen to preferentially detect a more slowly migrating form (V57').

presynaptic GABA_B receptors, to ethanol and to antipsychotic medications (Kang *et al.* 2003, Zink *et al.* 2004, Zink & Spanagel 2005). Since, in the pancreatic islets, GAD65 and GAD67 expression and GABA release are subject to metabolic regulation, we

hypothesized that islet VIAAT content may be similarly regulated (Hao *et al.* 1994, Smismans *et al.* 1997, Petersen *et al.* 1998). We therefore investigated VIAAT expression after islet exposure to different glycemic conditions. Rat islets were equilibrated overnight in

Figure 5 Effects of glucose concentration on islet VIAAT content. (A) Islets were isolated and allowed to equilibrate overnight in RPMI. Equal numbers of islets were then cultured separately at the glucose concentrations indicated, and V52 and V57 content was analyzed by immunoblotting with VT/510 and VT/75. Islet V57 content did not vary with glycemic conditions. V52 content varied as shown (levels shown are relative to V52 content at 3 mmol/l glucose; $n=6$; $P>0.05$ for the difference between the 10 mmol/l and 20 mmol/l glucose datasets). (B) We noted that islet V52 content decreased over 20 h after newly isolated islets were placed in culture. To test the effect of glucose on the decline in V52 levels during this initial 20-h period, newly isolated islets were cultured for 4 or 20 h after purification as indicated above each lane or lysed immediately after isolation (0 h, left-most lane). The culture media contained either 3 or 20 mM glucose, as indicated. After 4 or 20 h in culture media, the islets were harvested and lysed. VIAAT content in the resulting extracts was analyzed by immunoblotting. A representative experiment is shown. As previously noted, V52 content fell after the newly purified islets had been in culture for 20 h. Higher glucose concentrations attenuated this decrease. After 4 h, V52 content was 56% ($\pm 7\%$) greater in the islets cultured at 20 mmol/l glucose, and, after 20 h, V52 content was 41 ($\pm 5\%$) greater at the higher glucose level ($n=5$, $P<0.05$ for both). In contrast to V52 content, content of V57 and glyceraldehyde 3-phosphate dehydrogenase (G3 PDH) did not change significantly in response to removal of islets from the pancreatic to the tissue culture environments or in response to glycemic conditions. Note also that there was also no change in the protein apparent as a background band above the two VIAAT bands.

standard RPMI and then cultured for 20 h in different glucose concentrations. Afterwards, islet VIAAT content was examined by immunoblot analysis (Fig. 5A). V57 content did not vary in response to media glucose concentration. V52 levels, however, increased in response to higher glucose levels. Consistent with previous published results (Hao *et al.* 1994, Petersen *et al.* 1998), levels of GAD67 – the form of GAD present, along with VIAAT, in the islet mantle – were also observed to increase (data not shown).

Unexpectedly, we noted that V52 levels decreased relative to V57 levels during the overnight period of equilibration in RPMI medium immediately following islet isolation. The extent of decline was variable, and V52 levels reached their nadir – 15–30% of 4-h culture levels – by 20 h (Fig. 5B and data not shown). Elevated glucose concentrations significantly attenuated but did not prevent the fall in V52 content: after 4 h, V52 content was 56% ($\pm 7\%$) greater in islets cultured at

20 mmol/l glucose than in islets cultured in 3 mmol/l glucose, and, after 20 h, V52 content was 41% ($\pm 5\%$) greater at the higher glucose level ($n=5$, $P<0.05$ for both; see Fig. 5B). In contrast, V57 and glyceraldehyde 3-phosphate dehydrogenase levels did not change (Fig. 5B). During the initial 20-h period of equilibration in culture media after islet isolation, then, a higher glucose concentration resulted in less of a decline in V52 content (as in Fig. 5B), but the overall effect of glucose on V52 content was less than in experiments performed after the islets had been allowed to equilibrate in culture medium overnight (as in Fig. 5A).

VIAAT expression in INS-1 and islet β cells

Conflicting results have been published concerning whether VIAAT is expressed in β cells (Chessler *et al.* 2002, Hayashi *et al.* 2003a, Gammelsaeter *et al.* 2004). Immunoblot analysis showed that the rat β cell line

Figure 6 VIAAT expression in the β cell line INS-1. (A) Immunoblot of extracts prepared from rat islets (lane 1) and INS-1 cells (lane 2) using antibody VT/510. INS-1 cells express VIAAT, but only V57 and not V52. After INS-1 cell fractionation, V57 was detected in S2 (lane 4), consistent with its presence in synaptic-like microvesicles. VIAAT is also detected in P2 (lane 3) which contains the other membranous compartments, such as endosomes and secretory granules, separated from the microvesicles during the high-speed centrifugation. (B) INS-1 cells were either maintained in 11 mM glucose (lane 1) or switched 6 h (lanes 2 and 3) or 24 h (lanes 4 and 5) before being harvested into medium with 5 mM (lanes 2 and 4) or 25 mM (lanes 3 and 5) glucose. Equal amounts of extracted proteins were analyzed by SDS-PAGE with antibody VT/510. V57 content did not change in response to changes in glucose concentration. As a control for protein loading, the blot was simultaneously probed with an antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C) In parallel with the experiment shown in (B), insulin secretion was assessed in INS-1 cells cultured in low glucose (2.5 mM) or high glucose (25 mM) for 2 h. Secretion is shown as a percent of total cellular insulin content ($n=12$, $P<0.001$ for the difference between low and high glucose). (D) INS-1 cells were immunostained for insulin (INS, red) and VIAAT (with VT/510; VGAT, green). The merged image shows that VIAAT and insulin do not co-localize. Control staining of INS-1 cells (panel labeled IgG) with non-immune IgG revealed no non-specific immunofluorescent signal.

INS-1 expressed V57 but not V52, consistent with β cell expression of at least one form of the transporter (Fig. 6A) (Poitout *et al.* 1996).

Gammelsaeter *et al.* (2004) presented evidence that VIAAT localizes to synaptic-like microvesicles (SLMV) in β cells. To test whether VIAAT also associates with SLMVs in INS-1 cells, cells were fractionated as described in Materials and methods to yield a high-speed supernatant S2 containing microvesicles and depleted of secretory granules and endosomes (Clift-O'Grady *et al.* 1998). S2 contained VIAAT (Fig. 6A); this demonstrates its presence in SLMVs but

does not exclude that it is also present in secretory granules (Clift-O'Grady *et al.* 1990, 1998). Detection of microvesicular proteins in P2 in addition to S2 is typical due to their passage through other, larger membranous compartments, such as endosomes, during sorting. Because of this, detection of VIAAT in P2, as seen, is not informative as to whether VIAAT is also present in secretory granules (Fig. 6A). V57 content in INS-1 cells did not change in response to glycemic conditions (Fig. 6B); this was in contrast to insulin secretion, which was tested in parallel (Fig. 6C). Immunofluorescent staining revealed that INS-1 cells uniformly expressed

Figure 7 Immunostaining of rat and human pancreas sections. (A and C) VIAAT was detected in (A) rat and (C) human islets using antibody VT/75. In contrast to antibody VT/510, which previously yielded weak staining of the islet core (β cell region), with VT/75, detection of VIAAT in the islet core was readily apparent. These results were reproducible using a monoclonal antibody (M-VT/75) targeted to the same VIAAT peptide. (D) Human pancreatic tissue stained using M-VT/75. (B) Non-immune rabbit IgG was used in the same concentration as VT/75 as a control for non-specific staining. A representative rat islet (between arrows) stained with control IgG is shown.

both insulin and VIAAT (Fig. 6D). Consistent with trafficking of the transporter to microvesicles in INS-1 cells, VIAAT and insulin did not co-localize (Fig. 6D). The intranuclear staining seen with VT/510 was likely an artifact of cell fixation such as has been previously described (Chang *et al.* 1996, Wiczorek *et al.* 1997, Miller 2001).

Antibody VT/510 previously yielded relatively weak staining of the rat islet core (β cell region) compared with the mantle (Chessler *et al.* 2002). Because VT/75 displays a different pattern of recognition of the modified forms of V57 and because it has been proven to be an effective antibody for immunohistochemistry in a variety of settings (see, for example: Geigerseder *et al.* 2003, Prange *et al.* 2004, Chih *et al.* 2005, Saito *et al.* 2005), we used it to stain rat and human pancreas sections (Fig. 7). VT/75 made staining of the rat islet core readily apparent and enabled detection of VIAAT in human islets for the first time (Fig. 7A and C). These results were reproducible with M-VT/510, a monoclonal antibody made against the same peptide (for an example see Fig. 7D).

Discussion

Islet cells likely evolved from an ancestral neuronal precursor and have inherited a diverse set of proteins

that, in the central nervous system, provide the machinery for cell-to-cell signaling across synapses (Donev 1984, Regoli *et al.* 2000, Rulifson *et al.* 2002). These proteins include GAD65, GAD67, GABA receptors, glutamate and glycine receptors and vesicular glutamate and GABA transporters (Satin & Kinard 1998, Chessler *et al.* 2002, Hayashi *et al.* 2003b, Gammelsaeter *et al.* 2004). It is highly likely that, in the islet, these proteins are similarly constituents of intercellular signaling mechanisms. Continued characterization of the islet neurotransmitter signaling apparatus will be important for eventually understanding islet paracrine and autocrine signaling and the means by which islet function is co-ordinated at the cellular level.

Here, we have followed up on our prior work demonstrating that rat islets contain VIAAT, including a heretofore unidentified 52.5 kDa variant: V52. We have now identified V52 as an isoform of VIAAT that likely results from translation initiation at an alternate, downstream start site. V52 is subject to a different pattern of post-translational modifications than V57. Cellular V52 content is regulated independently of V57 content and is responsive to changes in glucose concentration. V52 content is affected as well by other, extracellular conditions, since basal levels changed during islet culture regardless of glycemic conditions. V57, we found, is subjected to a complex pattern of post-translational modifications. These modifications result in formation of a doublet, V57 and V57', seen on Western blots, with V57' being most evident in brain extracts (Bedet *et al.* 2000). The true heterogeneity of post-translationally modified V57, however, is only apparent after 2-D electrophoresis which, we have shown, reveals approximately five to eight differentially modified species.

It is clear that, in the rabbit reticulocyte lysate system, V52 is synthesized as a result of utilization of an alternate initiator codon. This mechanism for formation of multiple protein products from the same gene, while not common, has been observed with a variety of other eukaryotic genes (for example, see references in: Barraille *et al.* 1999, O'Donovan & Baraban 1999, Liu *et al.* 2000, Byrd *et al.* 2002). Rat islet V52 and *in vitro* synthesized V52 co-migrate by SDS-PAGE and by 2-D NEPHGE/SDS-PAGE, suggesting that they are identical and that they are thus formed by the same mechanism of downstream translational initiation. It is also possible that V52 is formed in islets by proteolytic cleavage during post-translational processing. In this case, the identical (or nearly identical) localization of an alternate initiation codon and a proteolytic cleavage site would have to be attributed to an unlikely coincidence. No evidence of a proteolytic activity that converts V57 to V52 was detected in rat islet extracts. Since antibody VT/17 binds only V57 while VT/75 binds both V52 and V57, the site of the amino-terminal truncation that

results in V52 must lie between the binding sites of the two antibodies. These reside between VIAAT residues 17–30 for VT/17 and between residues 75–87 for VT/75.

In the central nervous system, VIAAT traffics to synaptic vesicles and may associate with GAD65 (Jin *et al.* 2003). In islet α cells, GAD65 is absent, and the trafficking pattern of VIAAT is different, as the protein ends up associated predominantly with secretory granules (Hayashi *et al.* 2003b). It is possible that – as a result of truncation of VIAAT's cytoplasmic amino-terminal domain – V52 is better adapted to the different pathway of intracellular sorting and protein complex formation that is perhaps followed by VIAAT in the islet α cells. Here we have shown that, in rat islet extracts, up to eight species of modified V57 but only one of V52 are detected by 2-D NEPHGE/SDS-PAGE. Further work is needed to more fully characterize the sorting and post-translational modification pathways of the two isoforms.

Ebihara *et al.* (2003) have reported that mouse neurons synthesize a VIAAT splice variant with a unique carboxyl-terminal sequence. This splice variation, like the truncation that produces V52, affects the cytoplasmic region of the protein (McIntire *et al.* 1997, Ebihara *et al.* 2003). While the mouse VIAAT coding region spans three exons, the exon structure of the rat VIAAT gene is identical to the human exon structure: the amino-terminal 130 residues are encoded by one exon and the remaining 395 residues by a second (Chessler *et al.* 2002, Ebihara *et al.* 2003). In the absence of an exon–intron structure amenable to minor splice variations, as in the mouse, utilization of alternate initiator codons provides a different means for modification of the cytoplasmic region of VIAAT in rat and human cells. Others have presented evidence that protein expression can be regulated in this manner and have proposed mechanisms (Barraille *et al.* 1999, O'Donovan & Baraban 1999, Liu *et al.* 2000, Byrd *et al.* 2002).

Immunohistochemistry with antibody VT/510 suggested that rat islet β cells contain VIAAT (Chessler *et al.* 2002). Results published subsequently have been conflicting (Hayashi *et al.* 2003a, Gammelsaeter *et al.* 2004). The immunostaining results shown herein provide further evidence of β cell expression of VIAAT. We also found that INS-1 cells contain V57 but not V52. This result is consistent with the presence of VIAAT in the islet β cells and suggests that V52 may be limited to the α cells. The mechanism whereby V52 expression is suppressed in INS-1 cells remains to be determined. Regulation may occur at the level of translation, as has been described with other proteins formed from alternative start sites or, alternatively, there may be increased turnover of V52 in INS-1 cells (Barraille *et al.* 1999, Liu *et al.* 2000, Byrd *et al.* 2002).

The islet GAD–GABA system is subject to metabolic regulation. Levels of GAD65 and GAD67 expression and of GABA release vary in response to glycemic conditions (Hao *et al.* 1994, Smismans *et al.* 1997, Petersen *et al.* 1998). This suggests that GABA signaling may play a role in co-ordinating islet function to maintain euglycemia. Glycine likely plays a similar role (Gammelsaeter *et al.* 2004). Unlike neurons or islet β cells, rat α cells contain only GAD67: GAD65 is absent. This unique feature of the mantle GAD–GABA system has important implications, as it limits the means by which GABA signaling can be regulated. Unlike GAD65, which is highly regulated, both by association/dissociation with an essential cofactor and by phosphorylation, GAD67 activity is not actively regulated, and the enzyme is, in essence, constitutively active (Lernmark 1996, Battaglioli *et al.* 2003). We therefore hypothesized that regulation of α cell VIAAT content could provide an important means by which to regulate GABA release. Regulation of VIAAT levels would similarly be expected to modulate islet cell glycine release. Since GABA levels are markedly lower in rat α cells than in β cells, regulation of glycinergic signaling may be particularly relevant to α cell function (Gammelsaeter *et al.* 2004).

Our results have shown that cellular V52 content increases with glucose concentration. Interestingly, the α cell content of vesicular glutamate transporter (VGLUT) is affected by glycemic conditions in the opposite direction (Bai *et al.* 2003). Glutamate is an excitatory neurotransmitter and, like VIAAT, α cell VGLUT is secretory granule associated (Hayashi *et al.* 2003b). It is possible, therefore, that as glucose concentrations rise, α cells secrete relatively more GABA and glycine and less glutamate. Besides glycemic conditions, other metabolic or intercellular signals play an important role in determining islet V52, but not V57, content, as levels of the smaller isoform dropped after islets were removed from their native environment and placed in tissue culture. Regulation of cellular VIAAT content is not unique to islet cells, as neuronal VIAAT content responds markedly to agonists and antagonists of presynaptic GABA_B receptors, ethanol and antipsychotic medications (Kang *et al.* 2003, Zink *et al.* 2004, Zink & Spanagel 2005).

In summary, we have identified a novel isoform of VIAAT, V52, a variant of the transporter truncated within the cytoplasmic, amino-terminal domain. V52 is abundant in rat islets. In reticulocyte lysates, VIAAT cDNA directs the synthesis of both V52 and V57 as a result of utilization of alternate start sites of translation, and islet V52 is likely formed by the same mechanism. Post-translationally, V52 traverses a different pathway of processing and, perhaps, sorting than V57. Islet-derived V57, unlike V52, displays a complex pattern of post-translational modifications. By immunostaining

pancreas sections, we confirmed prior reports that VIAAT is present in islet β cells and detected the protein in both rat and human islets. We also found that the β cell line INS-1 expresses VIAAT, but only in the form of V57. Islet content of V52, but not of V57, increases in response to higher glucose concentrations. This is consistent with prior results indicating that the islet GAD–GABA system is responsive to metabolic stimuli. This sensitivity to metabolic stimuli, in turn, suggests that GABAergic and probably also glycinergic signaling help to co-ordinate the endocrine functioning of the islet.

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