J. Biochem. **142,** 633–638 (2007)
doi:10.1093/jb/mvm173

Expression of Glutamate Decarboxylase Isoform, GAD65, in Human Mononuclear Leucocytes: A Possible Implication of C-terminal End Deletion by Western Blot and RT–PCR Study

Satoko Matsukawa and Hiroshi Ueno*
Laboratory of Applied Microbiology & Biochemistry, Nara Women's University, Nara 630-8506, Japan

Received August 5, 2007; accepted August 29, 2007

Human peripheral blood leucocyte was examined for the expression of glutamate decarboxylase (GAD). Peripheral blood from healthy individuals was fractionated into polynuclear leucocytes and mononuclear leucocytes. Cell lysate from the mononuclear leucocytes was analysed by SDS–PAGE and Western blot. With antibody raised against unique C-terminal sequence for GAD65, two protein bands at 30 and 80 kDa were detected. However, with anti-GAD65/67 antibody recognizing very end of C-terminal, about 40 residues toward C-terminal, no protein bands were observed. Expression of mRNA coding for the epitope of these two antibodies was examined by using PCR technique. Results showed an evidence that mononuclear leucocytes express GAD65 with its C-terminal segment truncated. Our results have suggested an expression of GAD with the novel molecular weight may be caused by possible mononuclear leucocyte specific splicing errors.

Key words: GABA, GAD, leucocyte, splicing.

Abbreviations: DTT, dithiothreitol; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GAPDH, glyceraldehyde 3′-phosphate dehydrogenase; LiDS, lithium dodecyl sulphate; RNase, ribonuclease; RT-PCR, reverse transcriptase-polymerase chain reaction; TBS, Tris buffered saline; TCA, trichloroacetic acid.

Glutamate decarboxylase (GAD) [E.C.: 4.1.1.15] is an enzyme to synthesize γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in higher animals (*1*). GAD is widely distributed among the living cells of various organisms from mammalians to single cell organisms (*1*, *2*). Attempts have been made in order to explore the roles of GAD other than neurotransmitter. Recently, it has become clear that mammals have two distinct genes for GAD, GAD65 and GAD67 (*3–5*). Those two GAD isoforms respond differently to their common cofactor pyridoxal 5′-phosphate (PLP) and show distinct subcellular localization (*6*, *7*), which suggests that GAD isoforms may have different physiological roles. The distinct nature of GAD isoforms is found at their N-terminal 100 amino acid regions, where sequence homology is significantly lower than the remaining regions. GAD67 protein, a relatively hydrophilic and soluble polypeptide, is found mainly in the cell bodies of neurons and in the cytosol of other cells including pancreatic β-cells. GAD65 is somewhat more hydrophobic, less soluble than GAD67, and reversibly anchored to the membrane of synaptic vesicles of neurons and synaptic-like microvesicles in pancreatic β-cells (*6*). It has been suggested that GAD67 is responsible for most of the metabolic GABA synthesis in the brain (*7*, *8*). Obata and others have created knockout mice to examine functionality of GADs. The homogeneously knockout mouse, GAD67−/−, was born with cleft palate and died within a day after the birth, while the GAD65−/− knockout mouse is survived with a slightly increased tendency in having seizures (*8*).

*To whom correspondence should be addressed. Tel: +81-742-20-3493, Fax: +81-742-20-3448, E-mail: hueno@cc.nara-wu.ac.jp

Recently, GAD65 expressed in the pancreatic β-cells was identified as a target antigen protein for the autoantibody found in patients with-type I diabetes (*9*, *10*). In this case, GAD65 but not GAD67 is responsible for the activation of T cells. This indicates that the amino acid sequence or protein structure unique to GAD65 is involved in the activation, and the enzymatic activity of GAD is not. We have suspected if GAD65 may be expressed in the immune cells, and attempted to identify what sort of influence would be projected for the activation of T cells to raise autoantibody. To this extent, it is of interest for finding out if GAD65 is expressed in the immune-related cells.

The multi-functional nature of GAD is suggested by the recent findings, in which GAD localization was demonstrated in such tissues as skins, salivary gland, stomach, intestine and taste bud, all of which are not directly related to the neuronal functions (*11–14*). For many years, GAD expression was thought to be limited among brain, neuronal system, pancreas and testis, due to the high enzymatic activity found in the homogenates from these tissues. Recent advancements have allowed us to visualize protein expression within a single cell. By using RT–PCR and/or immunohistochemical methods with the use of fluorescent-labelled antibody, it has become possible to study the distribution of protein expression in most cells. In this study, we report the GAD65 expression in peripheral blood leucocytes.

MATERIALS AND METHODS

Western Blot Analysis—Blood sample was collected from healthy male donor. Separations of mononuclear cell and polynuclear cell were carried out by using

lymphoprep reagent (Daiichi-Kagaku, Japan) according to the manufacturer's instruction. Obtained mononuclear leucocytes and polynuclear cells were precipitated in 10% TCA for 30 min at 4°C. TCA-precipitated subcellular fraction was treated with lysis solution containing 8 M urea, 2% Triton X-100, 1 mM DTT and disrupted by ultrasonication, followed by an addition of 2% LiDS. As a positive control for GAD immuno-positive sample, frozen rat brain was used by homogenizing in Tris buffer at pH 7 containing 2% Triton X-100, disrupted by ultrasonication followed by precipitation with 20-fold volume of acetone for 30 min at −80°C. Mononuclear leucocytes were treated likewise to give acetone-precipitated fraction. Protein contents in each fraction were estimated as follows: aliquots of lysates were dot-blotted onto nitrocellulose membrane. The membrane was stained with Sypro Ruby (Invitrogen), fluorescent developed was captured by CCD camera and intensity was quantitated by comparing BSA standard spotted at known concentrations.

SDS-PAGE analysis with gels at 10–20% was carried out for the lysate containing 1 or 2 μg of protein and Protein ladder markers (XL-ladder broad, Aproscience). Resulting gels were electrotransferred onto PVDF membrane. After blocked in a 4% solution of Block Ace (Dainihon-Sumitomo, Japan), the membranes were incubated with rabbit anti-GAD65 antibody (Sigma G4913) or anti-GAD65/67 antibody (Sigma G5162) diluted at 1:2000 or 1:3000, respectively, in antibody reaction buffer containing 0.05% Tween-20 and 1% Block Ace in TBS. After the incubation with first antibody being carried out for overnight at 4°C, the membranes were washed 3 times with TBS containing 0.05% Tween-20 for 10 min. Then, the membranes were incubated for 2 h at 37°C with horseradish peroxidase-conjugated goat anti-rabbit antibody diluted 2000 times with antibody reaction buffer. After being washed three times, ECL plus reagent (GE Healthcare Lifescience) was added and immunoreactive proteins were visualized with an enhanced chemiluminescence detection system. Experiments were performed at least three times.

Analysis of PCR Products—Total RNA from mononuclear leucocytes of human blood (1×10^7 cells) was isolated by using Trizol reagent (Invitrogen). Further purification of RNA was carried out by using RNeasy kit (Qiagen). RNA was stored in sterile RNase-free water at −80°C as recommended by the supplier. Quantitation of the total RNA was done spectrophotometrically at 260 nm and quality of the isolated RNA was analysed with a Bioanalyzer (Agilent).

Conversion of mRNA to cDNA was carried out by using Omniscript reverse transcriptase kit (Qiagen). According to the manufacture's protocol, 300 ng RNA was mixed with 4 U of Omniscript reverse transcriptase, 10 U of RNase inhibitor, 20 pmol of oligo dT primer, 1 nmol of dNTP mixer and 1× BufRT in a final volume of 20 μl. This mixture was incubated at 37°C for 60 min. For a positive test, we have used human brain cDNA (Origine). By using the obtained cDNA as a template, reverse transcriptase-PCR (RT–PCR) was carried out. For comparison, we have monitored the expression levels of GAPDH for mononuclear leucocyte and that for human brain.

Table 1. **Description of primer sets.**

	Start	Length	tm	gc %	seq
Primer set 1					
Left primer	1526	20	60.02	50.00	ttcctccaagcttgcgtact
Right primer	1679	20	59.94	45.00	accatgcggaagaaattgac
Product size: 154					
Primer set 2					
Left primer	1526	20	60.02	50.00	ttcctccaagcttgcgtact
Right primer	1583	20	60.10	50.00	ttcgagaggcgactcattct
Product size: 58					
GAPDH primer set					
Left primer	251	19	61.65	47.37	ttccacccatggcaaattc
Right primer	420	20	62.11	55.00	tctccatggtggtgaagacg
Product size: 170					

Designing of PCR primers was basically carried out by using Primer 3 (for primer sets 1 and 2) or PCR Now (for GAPDH) web-based software as summarized in Table 1. Template cDNA sequences of human GAD65 and human GAPDH were obtained from GenBank (http://ncbi.nlm.nih.gov/). The access numbers for template cDNAs in GenBank are as follows: NM000818.1 for human GAD65 and NM002046 for human GAPDH. All primers designed by us were purchased from Invitrogen. PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) as follows: pre-denaturation at 95°C for 15 min and then amplification for 40 cycles, including denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR products were analysed by using Bio-Analyzer system (Agilent).

The product amplified from mononuclear leucocyte cDNA with primer set 2 was separated on 1.2% agarose gel (Wizard SV plus gel) electrophoresis and purified with PCR-clean up kit (Promega). The PCR products were then incorporated into pGEM vector and ligated by using pGEM-Teasy system (Promega). The integrated vector was transformed into JM109 strain, an *Escherichia coli* competent cell (Toyobo, Japan). The transformed *E. coli* cells were cultured on LB plate containing 100 μg/ml ampicillin at 37°C for overnight. Ten colonies were chosen for further culturing in liquid LB medium containing 100 μg/ml ampicilin at 37°C with shaking. After collecting the cells with centrifugation, plasmid was extracted by using Wizard Plus SV minipreps DNA purification systems (Promega). The isolated plasmid was sent to Shimadzu (Kyoto, Japan) for DNA sequence analysis where sequence primer M13-20 was employed.

RESULTS

Western Blot Analysis for GAD Protein—Protein expression study of GAD was investigated on mononuclear leucocytes and polynuclear leucocytes. Lysates from cell extracts were subjected for Western blot analysis where two antibodies, G5162 and 4913, were used for detecting GAD isoforms. According to

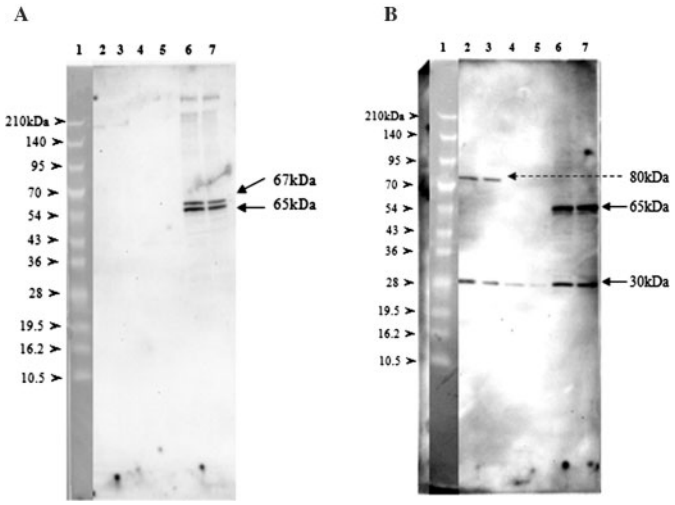

Fig. 1. **Western blot analysis of leucocyte lysates with anti-GAD antibodies.** Lysates from human mononuclear leucocyte and rat brain extract were separated on a SDS–PAGE, and then blotted to PVDF membrane. The membrane was immunostained with antibody: (A) anti-G5162 antibody and (B) anti-G4913 antibody. Lane 1, molecular ladder; lane 2, mononuclear leucocyte lysate 2 μg; lane 3, mononuclear leucocyte lysate 1 μg; lane 4, polynuclear leucocyte lysate 2 μg; lane 5, polynuclear leucocyte lysate 1 μg; lane 6, rat brain extract 2 μg; lane7, rat brain extract 1 μg.

manufacture, a synthetic peptide, KDIDFLIEEIERLGQDL, was used as an antigen for commercially available rabbit antibody G5162. This sequence corresponds to residues 570–585 locating at the very end of C-terminal region of human GAD65, and also to residues 579–594 at the end of C-terminal region of human GAD67. There is an extra Lys residue at the N-terminal which may not be affected to its antigenicity. It is noteworthy that residues 570–585 of GAD65 are highly homologous among mammalian species, thus, G5162 can recognize both GAD65 and 67 isoforms from human as well as rat. The rat brain extract gave two G5162 immunoreactive bands at 67 and 65 kDa (Fig. 1A, lanes 6 and 7). Under the same conditions, however, lysates from mononuclear leucocytes and polynuclear leucocytes showed no obvious immunoreactive band (Fig. 1A, lanes 2–5).

In case of antibody G4913 obtained from Sigma, rabbit was immunized with a synthetic peptide KRTLEDNEE RMSRLSKVA that corresponds to residues 514–530 of GAD65. This sequence is homologous in GAD65 from both human and rat, however it is not found in GAD67 from both species. Therefore, G4913 is thought to be specific to GAD65 from human as well as rat. The rat brain extract showed two immunoreactive bands estimated ~65 kDa and 30 kDa. The upper 65 kDa band seems to be the major one and 30 kDa band may be the minor one but is clearly visible (Fig. 1B, lanes 6 and 7). Lysates of mononuclear leucocytes exhibited two immunoreactive bands with estimated sizes of 80 and 30 kDa (Fig. 1B, lanes 2 and 3). For polynuclear leucocyte lysates, a very weak band at 30 kDa was evident but no detectable bands were observed at around 65 kDa or anywhere else (Fig. 1B, lanes 4 and 5).

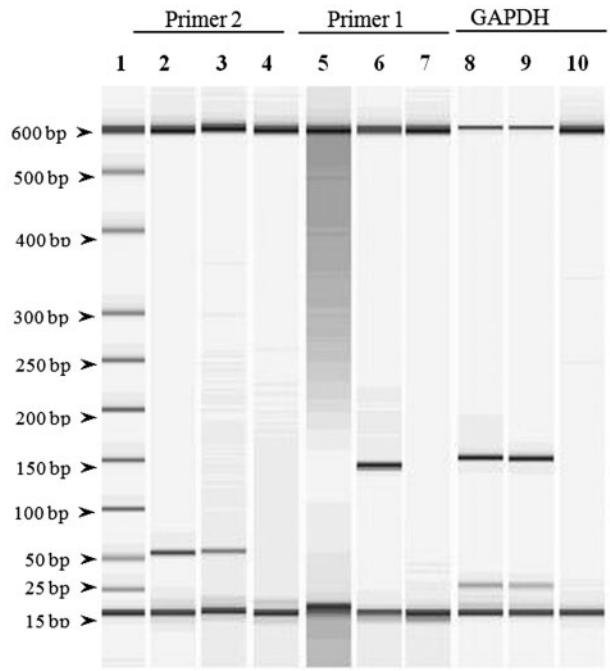

Fig. 2. **mRNA analysis on PCR.** PCR products were separated on a BioAnalyzer (Agilent). Lane 1 is a molecular ladder marker. Lanes 2–4, lanes 5–7 and lanes 8–10 were amplified with primer set 2, primer set 1, and GAPDH primer set, respectively. Lanes 2, 5 and 8 were for mononuclear leucocytes. Lanes 3, 6 and 9 are for rat brain. Lanes 4, 7 and 10 are controls containing no templates.

Analysis of Expression of mRNA Coding for GAD65 by PCR—After mRNA being converted into cDNA by the RT treatment, two sets of primers were used to amplify selective regions on GAD65 sequence (Table 1). Primer set 2 was designed to cover the epitope sequence for G4913 to give a 58 bp product. Primer set 1 was designed to overlap with primer set 2 and further extended toward the 3′ direction that ended at prior to the epitope sequence for G5162, giving a 154 bp product. For a positive control, GAPDH primer set was used. As shown in Fig. 2, both mononuclear leucocyte and rat brain exhibited a single band at around 150 bp, on lanes 8 and 9, respectively. Primer set 2 gave a single band at around the 50 bp marker for both mononuclear leucocyte and rat brain (Fig. 2, lanes 2 and 3). This result indicates that mRNA coding for the G4913 epitope was present in both mononuclear leucocyte and rat brain and also supports our Western blot analysis, in which G4913 antibody gave positive immunoreactive bands at 80, 65 and 30 kDa.

Similarly, we have amplified with the primer set 1 with mononuclear leucocyte and rat brain (Fig. 2, lanes 5 and 6). The rat brain sample gave a positive band at around 150 bp; however, there were no bands observed for the mononuclear leucocyte sample. Since the data presented here for both mononuclear leucocyte and rat brain have used the same successful preparations of mRNA and cDNA, we have explored a possibility of why the primer set 1 did not give any bands for mononuclear leucocyte. We have carried out DNA sequencing of

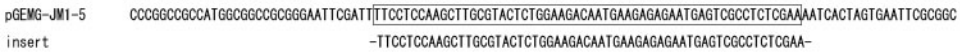

Fig. 3. **DNA sequence of the PCR product amplified mononuclear leucocyte cDNA with primer set 2.** PCR product was integrated into pGEM and analysed for the sequence. The sequence incorporated into pGEM is shown within the box. The insert sequence indicates the expected PCR product with primer set 2.

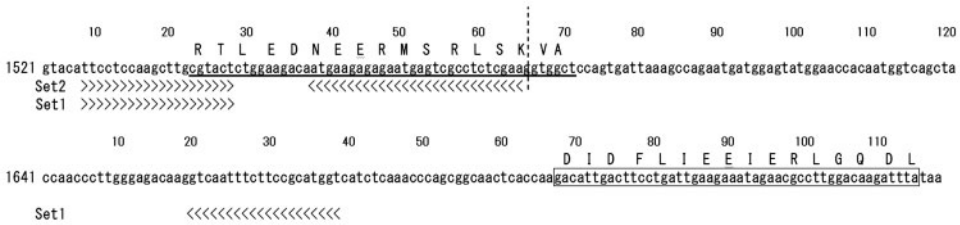

Fig. 4. **Location map of antigenic epitopes for G5162 and G4913 and PCR primer target sequences.** cDNA sequence at C-terminal of human GAD65 is presented with PCR primer sequences and antigenic epitopes for G5162 and G4913. The epitopes for G4913 and G5162 are underlined or shown with the box, respectively. The primer set 1 in the lower and set 2 in the upper are represented with > for the left and < for the right. The boarder of exon 15 and 16 is indicated by dotted line.

the band at 50 bp of mononuclear leucocyte in order to ensure that our PCR results did not pick up any contaminations to give a false-positive result. After the band was purified, it was incorporated into pGEM vector. By performing typical DNA mini-prep, the obtained vector was submitted to DNA sequencing. The results are shown in Fig. 3, in which the inserted sequence, as shown in lower lane, matched a GAD65 gene sequence of 1526–1583, 58 bp long, as indicated in Fig. 4. The sequence was exactly what we had expected for our PCR amplification with the primer set 2; thus, no contaminations were confirmed.

DISCUSSION

GAD is an enzyme to catalyse decarboxylation reaction of glutamate to produce GABA. GABA is an inhibitory neurotransmitter in higher animals; thus, GAD study is closely connected with that of GABA receptors. We have recognized the clinical importance of GAD in relation to the biological roles of GABA and its receptors. In early 90s, Baekkeskov *et al*. (*15*) reported that GAD65, one of the isoform of GAD, was found to be a target antigen for the autoantibody produced in patients with type I diabetes. Later, Yoon *et al*. have suggested that a specific part of GAD65 may activate T cell for antibody production that targets GAD65 expressed in the β-cells without having GAD enzymatic activities involved (*16*).

A comparative study of amino acid sequences for GADs indicates there are two distinct regions in GAD molecules, N-terminal 100 amino acid residues region and the remaining region. The N-terminal 100 amino acid residues are highly heterogeneous among isoforms, GAD65 and GAD67, if compared within the same species; however, they are homologous if each of the isoforms is compared among various animal species. The remaining region is the segment constituting decarboxylase function and it has high homology with other decarboxylases belonging to the same vitamin B_6 family, including aromatic amino acid decarboxylase and histidine decarboxylase(*2*). Another interesting characteristic on GAD is reported by Metzler and Chu (*16*) that the recombinant feline GAD67 expressed with its N-terminal 70 residues truncated has exhibited a full catalytic activity, which has suggested that N-terminal 100 amino acid residues may not be needed for the enzymatic activity but required for other biological functions, such as tissue specificity and subcellular localization.

Because of the above-described unique features built in GAD molecules and the fact that the two GAD isoforms catalyse the same chemical reaction and are expressed in the same cell, it is essential to perform careful mapping of GAD expression profiles in various tissues or cells. The specific localization of GAD and its isoforms has been examined on skins (*11*), salivary gland (*12*), stomach, intestine (*13*) and tongue (*14*); however, the expressions of GAD and its activity on those tissues had not been recognized. In the current study, we have focused on peripheral blood leucocytes because of the clinical interest on the mechanism of immune reaction with a possible GAD involvement. The Western blot analyses with G4913 antibody have shown that GAD65 immunoreactive bands are observed for mononuclear leucocyte lysates. Those GAD65 immunoreactive bands, however, exhibited at around 80 and 30 kDa, which are considerably larger and smaller, respectively, than what we have expected for GAD65, the mass of 65 kDa. There were no detectable bands for leucocytes when G5162 antibody was used.

The Western blot results as shown in Fig. 1 have implicated several interesting points. Human mononuclear leucocytes may express GAD65 at 80 kDa with degraded fragment at 30 kDa. The expression of GAD67 observable with G5162 antibody was not evident. The GAD65 bands detected with G4913 antibody must have a defective C-terminal end, which is an antibody recognition site for G5162, residues 570–585. We were unable to detect any bands corresponding to GAD67 with G4913 and G5162 antibodies. In theory, due to the site specificity of G4913 to GAD65 and G5162 to the C-terminal

segment common to GAD65 and 67, respectively, the GAD67 protein should be detected. Therefore, it is likely that GAD67 in the mononuclear leucocytes is missing its C-terminal end region. Since we did not have any suitable antibodies specific to GAD67, further experiments to seek GAD67 were not carried out.

The band at 30 kDa, a possible degradation fragment by protease(s), was only detectable by G4913 antibody. This fragment also lacked C-terminal end but had the C4913 recognition site. Therefore, a possible cleavage site is expected around the residue numbers of 200–220, and these numbers can be derived simply by counting 30 kDa toward to the N-terminal from the residue number 530 which is at the end of C4913 recognition site. We were not able to confirm the cleavage site, since our attempts to sequence this 30 kDa fragment were not successful due to the presence of contaminating proteins.

The band at 80 kDa is too large for GAD65, since its expected size from ORF is 65 kDa. We have considered a possibility of such post-translational modifications as phosphorylation, palmitoylation or glycosylation may have taken place. Bao *et al*. (*17*) and others have reported the phosphorylation form of GAD65, but their gel electrophoretic profiles did not show any bands around 80 kDa (*17–19*). The palmitoylation of GAD65 has been reported by several groups, however, none of the results suggest the formation of the large-size protein (*20*, *21*). Glycosylation on GAD65 has not been reported previously. Besides, obtaining the sharp electrophoretic band would be an unlikely event for that we deal with a glycoprotein. Therefore, we have concluded that the large molecular size observed for GAD65 is not due to the post-translational modifications. We have also considered possibilities of missplicing of mRNA and false-positive immunoreaction. Short forms of the enzyme such as GAD25 have been reported previously as a result of splicing variants (*22–24*). However, GAD25 is specifically observed for embryonic specimens and identified as a GAD67 variant. There has been no report for GAD65 variant. In addition, there is no known example for splicing variants giving larger molecular size in GAD. At present, we cannot explain why mononuclear leucocyte has 80 kDa form of GAD65 and this issue is left for our future studies.

We have carried out RT–PCR analysis on leucocytes with the two sets of designed primers, 1 and 2, to demonstrate if GAD65 is actually expressed at the mRNA level. Primer set 2 is designed to cover the range where G4913 antibody recognizes, whereas primer set 1 is extended further downstream approaching to G5162 antibody recognition site (Fig. 4). Amplification was successful for mononuclear leucocyte when the primer set 2 was used. We have isolated this amplified cDNA, inserted into pGEM vector, and then sequenced (Fig. 3). The results confirmed that the amplified DNA fragment contains the exact sequence as expected for the primer set 2 (Table 1). The primer set 1 failed to give any bands for the mononuclear leucocyte sample. These results indicate that the right side primer for the primer set 1 must have failed to hybridize to the target cDNA in case of mononuclear leucocyte. In GAD65 mRNA expressed in mononuclear leucocyte, a defect at the 5′-end is suggested. It is of interest to point out that there is an exon/intron boarder at the very end of the G4913 recognition site as shown in Fig. 4. If a deletion of the last exon in GAD65, exon 16, is occurred in mRNA level, both our Western blot and RT–PCR results could be explained. However, the deletion of the exon16 alone could not explain the presence of the GAD65 protein band at 80 kDa. It is possible to create such large molecular size protein if exons are utilized as the duplicated or a part of introns is remained. Further studies are needed to clarify this point.

REFERENCES

1. Roberts, E. and Frankel, S. (1951) Glutamic acid decarboxylase in brain. *J. Biol. Chem.* **188**, 789–795
2. Ueno, H. (2000) Enzymatic and structural aspects on glutamate decarboxylase. *J. Mol. Cataly. B.* **10**, 67–79
3. Erlander, M.G., Tillakaratne, N.J., Feldblum, S., Patel, N., and Tobin, A.J. (1991) Two genes encode distinct glutamate decarboxylases. *Neuron* **7**, 91–100
4. Karlsen, A.E., Hagopian, W.A., Grubin, C.E., Dube, S., Disteche, C.M., Adler, D.A., Barmeier, H., Mathewes, S., Grant, F.J., Foster, D., and Lemmark, A. (1991) Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. *Proc. Natl Acad. Sci. USA* **88**, 8337–8341
5. Bu, D.F., Erlander, M.G., Hitz, B.C., Tillakaratne, N.J., Kaufman, D.L., Wagner-McPherson, C.B., Evans, G.A., and Tobin, A.J. (1992) Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. *Proc. Natl Acad. Sci. USA* **89**, 2115–2119
6. Kaufman, D.L., Houser, C.R., and Tobin, A.J. (1991) Two forms of the γ-aminobutyric acid xynthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *J. Neurochem.* **56**, 720–723
7. Soghomonian, J.J. and Martin, D.L. (1998) Two isoforms of glutamate decarboxylase: why? *Trends Pharmacol. Sci.* **19**, 500–505
8. Asada, H., Kawamura, Y., Maruyama, K., Kume, H., Ding, R.-G., Ji, F.Y., Kanbara, N., Kuzume, H., Sanbo, M., Yagi, T., and Obata, K. (1996) Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. *Biochem. Biophys. Res. Commun.* **229**, 891–895
9. Baekkeskov, S., Kanaani, J., Jaume, J.C., and Kash, S. (2000) Does GAD have an unique role in triggering IDDM? *J. Autoimmun.* **15**, 279–286
10. Baekkeskov, S., Landin, M., Kristensen, J.K., Srikanta, S., Bruining, G.J., Mandrup-Poulsen, T., de Beaufort, C., Soeldner, J.S., Eisenbarth, G., Lindgren, F., Sundquist, G., and Lemmark, A. (1987) Antibodies to a 64,000 Mr human islet cell antigen precede the clinical onset of insulin-dependent diabetes. *J. Clin. Invest.* **79**, 916–934
11. Ito, K., Tanaka, K., Nishibe, Y., Hasegawa, J., and Ueno, H. (2007) GABA-synthesizing enzyme, GAD67, from dermal fibroblasts: evidence for a new skin function. *Biochim. Biophys. Acta* **1770**, 291–296
12. Iwahori, M., Akamatsu, K., Kurohara, S., Yokoigawa, K., Ueno, H., Ogawa, H., Ozawa, H., and Kawata, M. (2002) Immunohistochemical study of the localization of glutamate decarboxylase in rodent's submandibular gland. *J. Biol. Macromol.* **2**, 76–77
13. Akamatsu, K. (2004) Tissue localization study of glutamate decarboxylase in digestive system. Master's thesis, Nara Women's University; Akamatsu, K., Nakamura, Y., Hayasaki, H., Kanabara, K., Maemura, K., Yanagawa, Y., Obata, K., Watanabe, M., and Ueno, H. (2007) Cells expressing GABA synthetic enzyme, glutamate

decarboxylase, in stomach and intestine: RT-PCR and immunohistochemistry studies. *J. Biol. Macromol*., in press
14. Nakamura, Y., Yanagawa, Y., Obata, K., Watanabe, M., and Ueno, H. (2007) GABA is produced in taste bud. *Chem. Senses*, **32**, J19, http://chemse.oxfordjournals.org/cgi/reprint/32/2/J1
15. Baekkeskov, S., Aanstoot, H.J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H., and Camilli, P.D. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* **347**, 151–156
16. Chu, W.C. and Metzler, D.E. (1994) Enzymatically active truncated cat brain glutamate decarboxylase: expression, purification, and absorption spectrum. *Arch. Biochem. Biophys.* **313**, 287–295
17. Bao, J., Cheung, W.Y., and Wu, J.-Y. (1995) Brain L-glutamate decarboxylase. Inhibition by phosphorylation and activation by dephosphorylation. *J. Biol. Chem.* **270**, 6464–6467
18. Namchuk, M., Lindsay, L., Turck, C.W., Kanaani, J., and Baekkeskov, S. (1997) Phosphorylation of serine residues 3, 6, 10, and 13 distinguishes membrane anchored from soluble glutamic acid decarboxylase 65 and is restricted to glutamic acid decarboxylase 65α. *J. Biol. Chem.* **272**, 1548–1557
19. Wei, J., Davis, K.M., Wu, H., and Wu, J.Y. (2004) Protein phosphorylation of human brain glutamic acid decarboxylase (GAD)65 and GAD67 and its physiological implications. *Biochemistry* **43**, 6182–6189
20. Shi, Y., Veit, B., and Baekkeskov, S. (1994) Amino acid residues 24-31 but not palmitoylation of cysteines 30 and 45 are required for membrane anchoring of glutamic acid decarboxylase, GAD65. *J. Cell Biol.* **124**, 927–934
21. Solimena, M., Dirkx, R. Jr., Radzynski, M., Mundigl, O., and De Camilli, P. (1994) A signal located within amino acids 1-27 of GAD65 is required for its targeting to the Golgi complex region. *J. Cell Biol.* **126**, 331–341
22. Bosma, P.T., Blazquez, M., Collins, M.A., Bishop, J.D., Drouin, G., Priede, I.G., Docherty, K., and Trudeau, V.L. (1999) Multiplicity of glutamic acid decarboxylases (GAD) in vertebrates: molecular phylogeny and evidence for a new GAD paralog. *Mol. Biol. Evol.* **16**, 397–404
23. Chessler, S.D. and Lernmark, A. (2000) Alternative splicing of GAD67 results in the synthesis of a third form of glutamic-acid decarboxylase in human islets and other non-neural tissues. *J. Biol. Chem.* **275**, 5188–5192
24. Szabo, G., Katarova, Z., and Greenspan, R. (1994) Distinct protein forms are produced from alternatively spliced bicistronic glutamic acid decarboxylase mRNAs during development. *Mol. Cell. Biol.* **14**, 7535–7545