Alternative Splicing of GAD67 Results in the Synthesis of a Third Form of Glutamic-acid Decarboxylase in Human Islets and Other Non-neural Tissues*

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Two forms of glutamic-acid decarboxylase (GAD) have been identified in mammalian tissues: a 65-kDa form (GAD65) and a 67-kDa form (GAD67). Alternate splicing produces one or two smaller variants of GAD67 in the brain of embryonic mice and rats. Additionally, a short, heretofore unidentified transcript homologous to GAD67 has been detected in human testis RNA. Because GAD, the enzyme responsible for γ-aminobutyric acid production and a key autoantigen in type I diabetes, has unclear function in non-neural tissue, it is important to understand its pattern of expression. Unlike GAD65, GAD67 is not produced in human pancreatic islets. Here, we describe a novel splice variant of GAD67 that is produced in human islets, testis, adrenal cortex, and perhaps other endocrine tissues, but not in brain. This transcript directs the synthesis of a protein without GAD enzymatic activity: GAD25. A unique peptide sequence at the carboxyl terminus of GAD25 is highly conserved between mice, rats, and humans. We conclude that humans produce a third form of GAD in non-neural tissues and that human islets, although they do not synthesize full-length GAD67, do express this shortened variant.

Humans and other mammals synthesize two distinct forms of the enzyme glutamic-acid decarboxylase (GAD) (1, 2). One form, encoded by a gene on human chromosome 10, is a protein of ~65 kDa (GAD65). The other, encoded on human chromosome 1734, is ~67 kDa (GAD67). Both forms of the enzyme catalyze the formation of γ-aminobutyric acid from glutamate, although GAD67 has a markedly higher affinity for the cofactor pyridoxal 5'-phosphate, which is necessary for the activity of both forms (2, 3).

GAD expression is greatest in two tissues: brain and the pancreatic islets of Langerhans. Regardless of species, GAD65 and GAD67 are both abundant in brain, where γ-aminobutyric acid is a major inhibitory neurotransmitter (2, 4). In islets, the relative abundance of the two forms differs between species. In human islets, GAD65 is abundant, but immunocytochemistry, in situ hybridization, immunoprecipitation, and Western blotting have not detected GAD67. GAD67 message is not detectable in human islets by Northern blotting, although RT-PCR and RNase protection experiments suggest that there may be a low level of transcription (4–7). Monkey islets, like human islets, produce only GAD65 (8). In contrast, rat islets synthesize both isoforms, and mouse islets, which produce less GAD overall, produce predominantly GAD67 (5, 6, 9, 10).

Alternative splicing of GAD67 has been described in embryonic rodent brains. In mice and rats, an exon, which is itself alternatively spliced to either 80 or 86 base pairs (bp), is inserted into the full-length GAD67 message upstream of the pyridoxal 5'-phosphate-binding site in embryonic and fetal animals, but not in adult animals (11–13). The embryonic exon harbors an in-frame stop codon, resulting in the synthesis of a 25-kDa variant of GAD67, GAD25. If the exon is spliced to its 80-bp rather than its 86-bp form, another stop codon at the 3' end of the exon is removed, potentially enabling translation of a 44-kDa variant from a start codon within the embryonic exon (12).

GAD is also synthesized in testis. Here, although the 3.7-kb GAD67 message is detectable, the most abundant GAD transcript in humans is shorter, previously estimated to be ~2.5 kb (14). This small GAD transcript has not heretofore been identified, and it has not been certain whether it represents the product of a third GAD gene or a splice variant of one of the other two.

Here, we report the synthesis of a novel GAD transcript (a splice variant of GAD67) in human islet cells and testis. This transcript, which is also present in human adrenal cortex, is likely the previously unidentified short testis transcript. We show that the encoded protein, which lacks GAD enzymatic activity, is present in human islets and testis. This is the first report of a third variant of GAD in any human or other mammalian (non-endocrine) tissue and of the expression by human islets of a form of GAD other than GAD65.

**EXPERIMENTAL PROCEDURES**

**Tissues**—Human islet cells from nondiabetic adult organ donors were kind gifts from Dr. Daniel Pipeleers (β-Cell Transplant Central Unit, Vrije Universiteit Brussel, Brussels, Belgium) and Dr. Brian...
RESULTS

Detection of GAD Variants in Pancreatic Islets—As Northern blot analysis of islet RNA with a probe for GAD65 had previously revealed a 2.5-kb band (the putative transcript was referred to as “GAD3”) in addition to the expected 5.6-kb band (26, 27), we tested the hypothesis that a variant of GAD65 is synthesized in human islets. We initially probed Northern blots of monkey and human islet and pancreas RNAs with random-primed DNA probes and riboprobes for GAD65; washes were performed at various stringencies. Although detectable in some blots, the 2.5-kb band was not consistently reproducible, even under conditions in which there was cross-hybridization with GAD67 (data not shown).

As there is some evidence of autoreactivity to GAD67 in type 1 diabetes mellitus (20, 28–30), we speculated that a GAD67-like protein may be synthesized in human pancreatic islets. Because of this, we next utilized a probe specific for GAD67 for Northern blot analysis. The expected 3.7-kb band was seen in brain, but two unexpected, shorter bands (one ~1.5 kb and the other ~1 kb) were reproducibly observed in pancreatic RNA (Fig. 1).

Identification and Sequencing of a GAD67 Splice Variant (GAD67S)—These bands, representing shorter transcripts, pointed toward the existence of splice variants of GAD67 or possibly a third GAD gene. To identify possible splice variants or genes with high homology to GAD67, we searched EST sequences deposited in the GenBank™ Data Bank. Five human ESTs were identified that were homologous to GAD67 upstream of the codon for amino acid 213 (Met), but that diverged 3‘ of this site. Two of the ESTs were from a parathyroid tumor library (IMAGE clones 1405787 and 1341987), and the others were from testis, colon tumor, and breast tumor libraries (IMAGE clones 1644588, 1148313, and 1071440, respectively). Comparison of these ESTs to sequences in GenBank™ suggested that they derived from a single, novel transcript, most likely a splice variant of GAD67.

Primers were designed specific for this putative splice variant based on a consensus sequence derived from the ESTs. RT-PCR using these primers revealed that this transcript, which will be referred to as GAD67S, is produced in human testis and islets (Fig. 2). We were also able to amplify the expected GAD67S PCR product from monkey testis RNA, but not from RNA prepared from human breast tissue, human breast carcinoma tissue, or monkey brain (Fig. 2 and data not shown). The lengths of the RT-PCR products resulting from amplification using two different 5‘-primers specific for GAD67 upstream of the putative splice site and a 3‘- primer specific for GAD67S were consistent with our hypothesis that GAD67S represents a splice variant of GAD67 rather than a novel gene.

We sequenced GAD67S through the splice site to the poly(A) tail using 3‘-RACE. The cDNA sequence, which was identical
The site at which GAD67S diverges from GAD67 is the human homologue of the 5'-splice site utilized for insertion of the rodent brain embryonic exon (11–13). As shown in Fig. 3, homology between GAD67S and the rodent embryonic transcript continues downstream to the 3'-splice site of the embryonic exon (GenBank™ accession numbers M38351 and Z49977). Here, GAD67S becomes homologous to genomic mouse DNA that was previously sequenced 3' of the embryonic exon (GenBank™ accession number Z49977).

In contrast to the mouse genomic sequence, however, the transcribed human sequence encodes a polyadenylation signal (Fig. 3). The predicted protein (Fig. 3) is the human form of GAD25 (12). Of note, the carboxyl-terminal 11 amino acids (where GAD25 diverges from GAD67) are identical in mouse, rat, and human. A search of the data bases at the National Center for Biotechnology Information for other homologous DNA or protein sequences downstream of the alternative splice site yielded no matches, suggesting that this 11-residue peptide sequence is unique to GAD25.

GAD25 lacks the binding site for the cofactor pyridoxal 5'-phosphate, suggesting a lack of glutamate decarboxylase activity by the protein. Consistent with this, in an in vitro assay for GAD activity, GAD25 failed to catalyze the release of [1-14C]glutamate (Fig. 4).

**Tissue Distribution of GAD67S Expression**—We utilized Northern blot analysis to ascertain which human tissues produce GAD67S. Consistent with our RT-PCR results (Fig. 2), the transcript (determined to be ~1.5 kb) was detected in testis (Fig. 5A). Brain, the organ in which GAD67 is most abundant, did not synthesize GAD67S (Fig. 5A; see also Fig. 1), although the probe did detect GAD67 (4). Adrenal cortex, a site of low level GAD67 transcription, produced GAD67S in greater abundance than testis (Fig. 5A)(4). Northern blot analysis of human pancreatic islet RNA (Fig. 5B) confirmed that the message was transcribed in these cells. Based on immunoblotting results (see below), we did not expect to find the message in monkey islets. However, it was detectable, although at levels ~4-fold less than in human islets. The message was not detected in the other tissues tested.

**The Protein Product of GAD67S, GAD25, Is Present in Human Islets and Testis**—Western blot analysis was employed to test for the presence of the GAD67S protein product in different tissues. To detect the protein, we used antisera raised against a synthetic peptide consisting of the amino-terminal 18 amino acid residues of GAD67/GAD25 (excepting the initiating methionine) (9). We detected GAD25 in human islet and testis extracts, but not in rat brain or monkey or rat islets (Fig. 6). GAD67 was present in rat brain extract, but consistent with previously published results (5, 7), not in human islets (Fig. 6C). As the primary and secondary antibodies were both polyclonal, immunoblotting produced a significant number of non-specific bands. To ensure that the ~25- and ~67-kDa bands represented GAD25 and GAD67, we demonstrated that we could specifically block antibody binding and detection of these bands by blocking the primary antibody with the GAD67/GAD25 amino-terminal peptide. A control peptide with amino-
FIG. 5. GAD67S is transcribed in human testis, adrenal cortex, and islets, but not in brain. A, a commercially produced multitissue Northern blot made using human poly(A)–selected RNA was hybridized with a probe specific for GAD67S (and separately, one specific for actin). The 1.5-kb GAD67S band was apparent in the adrenal cortex and testis lanes, but not in the other lanes shown, including brain (which was on a separate membrane that was hybridized and washed in tandem). The 5′-portion of the probe contained sequence upstream of the splice site, resulting in hybridization to GAD67 message (brain lane). GAD67 message was not detected in testis on the blot depicted; the band may be obscured by the dark artifact at ~4 kb. B, total RNA from human islets (lane 1, 8 μg) and monkey islets (lane 2, 30 μg; and lane 3, 8 μg) was hybridized to the GAD67S probe in tandem with the blots shown in A. This blot was also separately probed for GAD65. The GAD67S band had approximately equal intensity in the human islet lane (lane 1) and the lane with more monkey islet RNA (lane 2). The 18 S ribosomal RNA band was detected using ethidium bromide and photographed under UV light prior to transfer to the membrane.

terminal GAD65 sequence did not block detection of the two bands (Fig. 6, B and C).

DISCUSSION

Unlike rodent islets, human islets are presumed to synthesise only GAD65, not GAD67 (2, 3). Neither GAD65 nor GAD67 variants have been described in the islets of either species. Here, we have shown that a short form of GAD, encoded by the novel transcript GAD67S, is expressed in human islets. GAD67S is a splice variant of GAD67. The encoded protein, GAD25, has been detected previously only in embryonic and fetal mouse brain, though it is likely also synthesized in rat embryonic brain (11, 12).

Knowledge of the pattern of GAD gene expression in human islets is essential, as GAD is a key and possibly the triggering autoantigen in type 1 diabetes mellitus, a disease resulting from autoimmune destruction of the insulin-producing β cells within the islets (2, 3, 15, 16). Although autoantibodies and T cell reactivity to GAD67 have been detected in patients with type 1 diabetes, this evidence of autoimmunity is generally attributed to cross-reactivity by autoantibodies and T cells reactive to GAD65. The reasons that GAD65 and not GAD67 is thought to function as an autoantigen in type 1 diabetes are 3-fold: first, because GAD65 autoantibodies are much more prevalent in patients with new-onset diabetes (70–90% versus ~10%); second, because GAD67 autoreactivity most often occurs in patients who also exhibit autoreactivity to GAD65; and, finally, because GAD67, unlike GAD65, is thought not to be synthesized in human islet cells (2, 3, 7, 17, 18). Our results show, however, that although these cells do indeed lack GAD67, human islets produce a truncated variant: GAD25.

A common feature of the major antigens targeted by autoantibodies in patients with type 1 diabetes is their direct association with the β cell secretory apparatus (3). It is thus interesting that the GAD67 amino-terminal sequences that may mediate association with GAD65 (and thus with the membrane of the islet synaptic-like microvesicle) are preserved in GAD25 (2, 3). In light of evidence from the non-obese diabetic mouse model of autoimmune diabetes that autoreactivity to GAD67 may help propagate or initiate islet cell destruction, it will be important to determine whether GAD25 plays a role in the pathogenesis of the disease in humans (2, 3, 15, 16). Presently, determination of whether there is T cell reactivity to unique GAD25 epitopes is hindered by the fact that reliable T cell assays for human autoreactive T cells have yet to be developed (31). Also, although we have detected a low prevalence of GAD25-specific humoral autoimmunity in patients newly diagnosed with type 1 diabetes, 2 determination of whether autoantibodies targeted to GAD25 are present early in the disease process, around the time of onset of islet cell autoimmunity, or are involved in the pathogenesis of other autoimmune endocrine diseases will require further study.

We have likely identified the heretofore uncharacterized short GAD67 testis transcript. Prior Northern blot analysis of human testis RNA revealed the expected 3.7-kb transcript, but also a more abundant, shorter message. Since the probe employed in these earlier studies included the entire coding region of GAD67, it would have hybridized to GAD67S (14, 32). The shorter GAD message was estimated to be ~2.5 kb (~1 kb longer than GAD67S), but the basis of that estimate is unclear. If there is a third, 2.5-kb GAD67–like transcript in testis, it is unclear why all three messages were not detected. At the time of writing, there was no evidence in the GenBankTM Data Bank of other human GAD67 splice variants, although one possible

2 S. D. Chessler, L. Bekris, and Lernmark, A., unpublished observations.
Molecular Characterization of a Novel Human GAD Transcript

Islet Isolation and Cell Processing Facility, Puget Sound Blood Center/Islets prepared by the assistance with tissue procurement. This study made use of human and Lisa Hammerle for assistance with the GAD activity assay. The study was conducted by Ben Snyder (University of Washington Diabetes and Endocrinology Center).

A key finding that has contributed to our current understanding of GAD function in general and the role of autoreactivity to GAD in diabetes mellitus has been the absence of GAD in human islets. In contrast, the transcript was not detected in brain. It is interesting that synthesis of this transcript may be confined to endocrine organs. An increased incidence of autoreactivity to GAD67 has been noted in association with autoimmune polyglandular syndrome type II, which commonly involves the adrenal cortex, islets (diabetes mellitus), and gonads (18). There is evidence of homology to other proteins. One may speculate that the carboxyl-terminal 11 amino acids are perfectly conserved between humans, rats, and mice, it is unclear what functionality this short peptide sequence might confer upon the enzyme, we could find no evidence of homology to other proteins. One may speculate that alternative splicing of the GAD67 message is possibly a means to down-regulate expression of the enzyme, but such alternate splicing, resulting in the synthesis of a 25-kDa protein, would be a surprisingly inefficient way to decrease GAD67 synthesis, and it would not explain the addition of a conserved stretch of amino acids to the truncated protein.

In addition to islets and testis, GAD67S is produced in adrenal cortex. Low level transcription of GAD67 (but not GAD65) has previously been detected in this organ (4). Two of the GAD67S ESTs we found were derived from a parathyroid library, suggesting that GAD67S is produced in that tissue as well. In contrast, the transcript was not detected in brain. It is interesting that synthesis of this transcript may be confined to endocrine organs. An increased incidence of autoreactivity to GAD67 has been noted in association with autoimmune polyglandular syndrome type II, which commonly involves the adrenal cortex, islets (diabetes mellitus), and gonads (18). GAD67S is the only form of GAD that has previously been detected in this organ (4). Two of the GAD67 ESTs we found were derived from a parathyroid library, suggesting that GAD67S is produced in that tissue as well.

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REFERENCES

1. Bosma, P. T., Blazquez, M., Collins, M. A., Bishop, J. D., Drouin, G., Priede, I. G., Docherty, K., and Trudeau, V. L. (1999) Mol. Biol. Ecol. 16, 397–404
2. Lernmark, A. (1996) J. Intern. Med. 239, 259–277
3. Melmed, S. (1998) J. Clin. Endocrinol. Metab. 14, 237–240
4. Malley, M. I., Cirulli, V., Otonkoski, T., Soto, G., and Hayek, A. (1996) Diabetes 45, 496–501
5. Kim, J., Richter, W., Aanstoot, H. J., Shi, Y., Fu, Q., Rajotte, R., Warnock, G., and Baekkeskov, S. (1993) Diabetes 42, 1799–1808
6. Petersen, J. S., Russel, S., Marshall, M. O., Kofod, H., Buschard, K., Cambon, N., Karlsen, A. E., Boel, E., Hagopian, W. A., Heijne, K. R., Moody, A., Dyberg, T., Lernmark, Å., Madsen, O. D., and Michelsen, B. K. (1995) Diabetes 44, 484–495
7. Cram, D. S., Faulkner-Jones, B., Kun, J., and Harrison, L. C. (1995) Endocrinology 131, 1111–1119
8. Hagopian, W. A., Karlsen, A. E., Petersen, J. S., Teague, J., Gervasi, A., Jiang, J., Fujimoto, W., and Lernmark, A. (1993) Endocrinology 132, 151–141
9. Li, L., Jiang, J., Hagopian, W. A., Karlsen, A. E., Skelly, M., Baskin, D. G., and Lernmark, A. (1995) J. Histochem. Cytochem. 43, 53–59
10. Faulkner-Jones, B. E., Cram, D. S., Kun, J., and Harrison, L. C. (1993) Endocrinology 130, 1773–1782
11. Bond, R. W., Wyborski, R. J., and Gottlieb, D. I. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8771–8775
12. Szabo, G., Katarova, Z., and Greenspan, R. (1994) Mol. Cell. Biol. 14, 7535–7545
13. Szabo, G., Katarova, Z., Kortvely, E., Greenspan, R. J., and Urban, Z. (1996) DNA Cell Biol. 15, 1081–1091
14. Persson, H., Pelto-Huikko, M., Metsis, M., Soder, O., Brenes, S., Skog, S., Hokfelt, T., and Ritzen, E. M. (1990) Mol. Cell. Biol. 10, 4701–4711
15. Yoon, J. W., Yoon, C. S., Lim, H. W., Huang, Q. Q., Kang, Y., Pyun, K. H., Kim, J. S., Kim, H. J., Choe, H. S., Heon, H. M., and Lee, E. H. (1999) Science 284, 1183–1187
16. von Boehmer, H., and Sarukhan, A. (1999) Science 284, 1135–1137
17. Seissler, J., Amann, J., Mauch, L., Hausruck, H., Woflaerts, S., Bie, S., Richter, W., Hall, R., Heinez, E., Northemann, W., and Scherbaum, W. A. (1993) J. Clin. Invest. 92, 1394–1399
18. Seissler, J., Bie, S., Yassin, N., Mauch, L., Northemann, W., Boehm, B. O., and Scherbaum, W. A. (1994) Autoimmunity 19, 231–238
19. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8998–9002
20. Gruhn, C. E., Daniels, T., Toivola, B., Landin-Olsson, M., Hagopian, W. A., Li, L., Karlsen, A. E., Boel, E., Michaelson, B., and Lernmark, A. (1994) Diabetes 43, 553–560
21. Madden, T. L., Tatsuvos, R. L., and Zhang, J. (1996) Methods Enzymol. 266, 399–405
22. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
23. Krieger, N. R., and Heller, J. S. (1979) J. Neurochem. 33, 299–302
24. Miller, L. P., Martin, D. L., Marzander, A., and Wallets, J. R. (1978) J. Neurochem. 30, 361–369
25. Ausubel, F. M. (1994) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
26. Edelhoff, S., Gruhn, C. E., Karlsen, A. E., Alder, D. A., Foster, D., Distech, C. C., and Lernmark, A. (1993) Genomics 17, 93–97
27. Karlsen, A. E., Hagopian, W. A., Gruhn, C. E., Boel, E., Distech, C. C., Adler, D. A., Barrie, H., Mathews, E., Grant, P. J., Foster, D., and Lernmark, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8337–8341
28. Honeyman, M. C., Cram, D. S., and Harrison, L. C. (1993) J. Exp. Med. 177, 535–540
29. Kaufman, D. L., Erlander, M. G., Clarke, S., Atkinson, M. A., Endert, P. M., Gottlieb, P. A., Wilson, S. B., and Sachs, J. A. (1999) J. Autoimmun. 13, 267–282
30. Kobayashi, Y., Kaufman, D. L., and Tobin, A. J. (1987) J. Neurosci. 7, 2768–2772

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