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Cloning, characterization, and autoimmune recognition of rat islet glutamic acid decarboxylase in insulin-dependent diabetes mellitus

(partial human insulinoma cDNA sequence/allelic variation/alternative mRNA processing/evolution)

BIRGITTE KOCH MICHELSEN*, JACOB STEN PETERSEN*, ESPER BOEL†, ANNETTE MØLDRUP*, THOMAS DYRBERG‡, AND OLE DRAGSBÆK MADSEN*

*Hagedorn Research Laboratory, Niels Steensensvej 6, DK-2820 Gentofte, Denmark; and †Bioscience, ‡Diabetes Research, Novo Nordisk A/S, Novo Alle, DK-2880 Bagsværd, Denmark

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ABSTRACT **A 64-kDa islet protein is a major autoantigen in insulin-dependent diabetes mellitus (IDDM). Autoantibodies against the 64-kDa protein were recently shown to immunoprecipitate glutamic acid decarboxylase (GAD; L-glutamate 1-carboxy-lyase, EC 4.1.1.15) from brain and from islets. We present evidence that the autoantisera also recognize a hydrophilic islet protein of ≈67 kDa in addition to the amphiphilic 64-kDa form. We have isolated a full-length rat islet GAD cDNA encoding a hydrophilic 67-kDa protein, which appears to be identical to rat brain 67-kDa GAD. A partial sequence of human insulinoma 67-kDa GAD was identical to human brain 67-kDa GAD. Allelic variations were observed in rat as well as in human 67-kDa GAD sequences. The expressed rat islet 67-kDa GAD protein is functional and is immunoprecipitated by IDDM sera; it comigrates electrophoretically with the 67-kDa islet autoantigen. The hydrophilic 67-kDa form of GAD in islets is an additional autoantigen in IDDM and is recognized by a different subset of autoantibodies than the 64-kDa autoantigen. Thus, mammalian cell lines expressing functionally active, recombinant GAD may become important tools to study the nature and the role of GAD autoreactivity in IDDM.**

Insulin-dependent diabetes mellitus (IDDM) is thought to develop as a result of autoimmune destruction of the islet β cells (1). Autoantibodies directed against an islet cell protein with a mass of 64 kDa have been detected in 80% of recent-onset IDDM patients, as well as in prediabetic individuals several years before clinical onset of the disease (1). Recently, it was demonstrated that the 64-kDa islet cell autoantigen is the enzyme glutamic acid decarboxylase (GAD; L-glutamate 1-carboxy-lyase, EC 4.1.1.15) (2), which is expressed in islets as well as in the brain (3).

Sera from IDDM patients immunoprecipitate a GAD molecule of ≈64 kDa (GAD64) from the Triton X-114 phase of rat islets and brain (2). The same molecule can also be immunoprecipitated from the aqueous phase along with a component of ≈67 kDa (designated 65K in ref. 2). It is not known whether this molecule is specifically recognized by the autoantibodies or whether it is coprecipitated due to interaction with the GAD64 or other molecules.

In the brain, different forms of GAD catalyze the biosynthesis of γ-aminobutyric acid (GABA) (4–7). By immunological techniques, two proteins have been identified, one of 67 kDa (GAD67) and one of 59–65 kDa (hereafter referred to as GAD64) (4–7), which differ in intracellular distribution and in the degree of saturation with the coenzyme, pyridoxal phosphate (PLP) (8). Immunoprecipitation experiments with antibodies against either brain GAD67 or GAD64 suggested that they may form heterodimers (7, 8).

The interrelationship between the GAD64 and the GAD67 forms in the brain is unknown, and their mutual relationship to the islet GAD autoantigen(s) has not been clearly established. To address this issue, we therefore isolated a GAD cDNA from rat islets and human insulinoma, determined its sequence, and studied the rat islet protein by functional expression in mammalian cells.§

MATERIALS AND METHODS

Rat Islet cDNA Library. Total RNA was isolated from 50,000 rat islets by CsCl gradient centrifugation (9). mRNA was purified on a poly(U) Sephadex column (Pharmacia) (10). First-strand cDNA was primed with oligo(dT) (Pharmacia) and synthesized with avian myeloblastosis virus reverse transcriptase (Life Sciences, Johannesburg, FL) (11) and second-strand synthesis with RNase H and DNA polymerase I (GIBCO/BRL) was performed as described (12). *Bst*XI adaptors (Invitrogen, San Diego) were added to the cDNA (13), which was then fractionated according to size on a potassium acetate gradient (14). The fractionated cDNA products were ethanol precipitated before ligation into the *Bst*XI site of pCDM8 (14). Ligated material was precipitated with ammonium acetate and ethanol (15) and used to transform *Escherichia coli* MC1061/p3 by electroporation (16).

Isolation of Rat GAD cDNA Clones. GAD-containing cDNA clones were recognized by colony hybridization (17, 18) using a synthetic GAD67-specific oligonucleotide probe: 5′-GGCAAAAATTCTTGATGCCAAACAAAAGGG-3′ (19). The identity of the putative GAD clones was confirmed by DNA sequencing using dideoxynucleotide sequencing kits (Pharmacia). All computerized sequence analyses and manipulations were performed with software from the University of Wisconsin Genetics Computer Group (20).

Human Insulinoma GAD cDNA Sequence. Total RNA was extracted (9) from a human insulinoma and primed with oligo(dT) in a cDNA synthesis reaction using the Moloney murine leukemia virus RNase H⁻ reverse transcriptase (GIBCO/BRL) as described by the supplier. PCR amplification with recombinant *Taq* DNA polymerase (Perkin–Elmer/Cetus) was performed on this cDNA with oligonucleotide primers specific for the feline brain GAD67 (21) (5′-CCGAAAGCTTGATTTTGATAAAGTGCAATGAAAGGG-3′) and the human male germ cell GAD (5′-TGC GTCTAGATTACAGATCCTGGCCCAGTCTTTC-3′) (22). *Hin*dIII and *Xba* I sites were introduced into these primers, which were synthesized on an Applied Biosystems 391 PCR-

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Abbreviations: GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; PLP, pyridoxal phosphate; GABA, γ-aminobutyric acid.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M76177).

Mate EP. An amplified 0.9-kilobase fragment was gel purified and cloned in pUC vectors (23) and sequenced (24).

RNase Protection Assay. The following probes were used: a rat islet GAD67 cDNA clone in pCDM8 was truncated downstream from a *Hin*dIII site 3′ to the translational stop codon. This construct was linearized with *Acc* I. Cyclophilin cDNA (25) subcloned into pGEM (Promega) was linearized with *Nco* I. Radiolabeled ([^{32}P]UTP; 3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) antisense RNA transcripts were generated from 1 μg of the linearized plasmids using T7 or SP6 RNA polymerase (Promega). The RNase protection assay was performed with a commercially available kit (Ambio, Austin, TX).

Expression of cDNA Clones in COS Cells. GAD clones with cDNA inserts in the sense ($pGAD_{sense}$) or antisense ($pGAD_{\alpha\text{-sense}}$) orientation with respect to the cytomegalovirus promoter in pCDM8 were transfected into COS-7 fibroblasts by the calcium phosphate precipitation technique (26), where 10^7 cells were seeded per 400-cm^2 tissue culture dish with 50 ml of Dulbecco's modified Eagle's medium (GIBCO)/50 μg of gentamycin per ml/110 μg of sodium pyruvate per ml/10% heat-inactivated fetal calf serum (GIBCO), and incubated at 37°C overnight. In 5 ml of 250 mM $CaCl_2$, 800 μg of $pGAD_{sense}$ or $pGAD_{\alpha\text{-sense}}$ was gently mixed with an equal vol of 2× HBS (0.82% NaCl/0.59% Hepes/0.02% Na_2HPO_4, pH 7.12) and added to each dish in addition to 1 ml of 10 mM chloroquin disphosphate. The cells were left with the DNA precipitate overnight, after which the medium was changed to culture medium.

Cellular Extraction. Cells were extracted by homogenization in hypotonic buffer (2) followed by centrifugation at 100,000 × *g* for 1 hr to obtain S-100 and P-100 fractions. The cytosol fractions of rat islet or COS-7 cell homogenates were further fractionated by Triton X-114 phase separation as described (2, 27, 28).

GAD Enzyme Activity Assay. GAD enzyme activities were measured in S-100, P-100, or crude extract as described (2).

Sera and Antibodies. The S3 antiserum to rat GAD was provided through the Laboratory of Clinical Science (National Institute of Mental Health) where it was developed under the supervision of Irwin J. Kopin with Wolfgang Oertel, Donald E. Schmechel, and Marcel Tappaz. The GAD67 antiserum 1266 was raised in rabbits immunized with the synthetic C-terminal sequence (Thr-Gln-Ser-Asp-Ile-Asp-Phe-Leu-Ile-Glu-Glu-Ile-Glu-Arg-Leu-Gly-Gln-Asp-Leu) as described (28). Rabbit antiserum to GABA was from Immunotech (Marseille, France). The IDDM sera from recent onset IDDM patients were provided by M. O. Marshall (Novo Nordisk A/S, Denmark). Twenty sera were tested for the presence of antibodies against the hydrophobic, small molecular mass (64 kDa) form of GAD. Ten of the positive sera were tested for reactivity to the rat recombinant GAD67 from transfected COS-7 cells. Control sera were random samples from 10 nondiabetic individuals.

Immunoprecipitation Assays. The aqueous phase or detergent phase of [^{35}S]methionine-labeled cell extracts was analyzed by standard immunoprecipitation methods (2, 29) using 400 islets or 2.5 × 10^5 transfected COS cells per assay. Immunoprecipitates were analyzed by $NaDodSO_4$/PAGE (10%) followed by electroblotting onto nitrocellulose and immunostaining (28) using the C-terminal GAD67 peptide antiserum 1266. Stained immunoprecipitation blots were autoradiographed.

Immunocytochemistry. Two-color double immunofluorescence labeling was carried out on fixed (1% paraformaldehyde, neutral) monolayers of transfected COS-7 cells to test the colocalization of the immunoreactivities of the brain GAD antiserum S3 and the C-terminal GAD67 antiserum 1266, as well as of GAD and GABA. Fluorescein isothiocyanate-conjugated donkey anti-sheep IgG (1:50 dilution; Chemicon) was used to visualize S3 binding and Texas Red-conjugated goat anti-rabbit IgG (1:100 dilution; Axell, Westbury, NY) was used to detect primary antibodies 1266 and anti-GABA. Species-specific secondary antibodies were mixed before the final incubation.

RESULTS

Isolation and Characterization of a Rat Islet GAD67 cDNA Clone. A rat islet cDNA library was screened with a synthetic oligonucleotide sequence based on the rat brain GAD cDNA sequences (19, 30). One of the clones thus isolated contained a full-length GAD cDNA of ≈3.7 kilobases, which is the size reported for the major transcript in brain tissue (22, 31, 32). The cDNA was shown to contain an open reading frame corresponding to that of the previously published rat brain GAD. The size of the encoded GAD polypeptide was calculated to be 66.6 kDa and is hereafter referred to as GAD67.

Allelic Variation of Rat GAD67. Alignment of two rat brain GAD67 cDNA sequences (19, 30) (Fig. 1, rb1GAD and rb2GAD), an alternatively spliced transcript from embryonic rat brain (33) and the rat islet cDNA (riGAD), revealed an allelic polymorphism, which encompasses at least three different alleles of the rat GAD67 gene. Most divergent are rb1GAD and rb2GAD, which differ from each other by 16 nucleotides, whereas riGAD is distinguished from rb1GAD and rb2GAD by 13 and 3 nucleotides, respectively (Fig. 1). Since the 3 nucleotide substitutions that discriminate riGAD from rb2GAD are silent, these two alleles are identical at the protein level (Fig. 2).

Allelic Variation of Human GAD67. Human islet and brain GAD67 sequences have been suggested to differ based on partial sequences (34). However, the two tissues may not be from the same donor since the islet cDNA and brain mRNA originated from the United States and Australia, respectively. Comparison of the partial amino acid sequence of the human insulinoma GAD67 with these GAD67 sequences (Fig. 2) demonstrated that the insulinoma sequence is identical to the human brain GAD67 but different from the human islet GAD67 sequence (34). These data indicate individual differences rather than tissue specificity of GAD67.

Differential Splicing of GAD67 mRNA in Human Insulinoma Tissue. During the characterization of the cloned human insulinoma GAD67 cDNAs, we also isolated a cDNA repre-

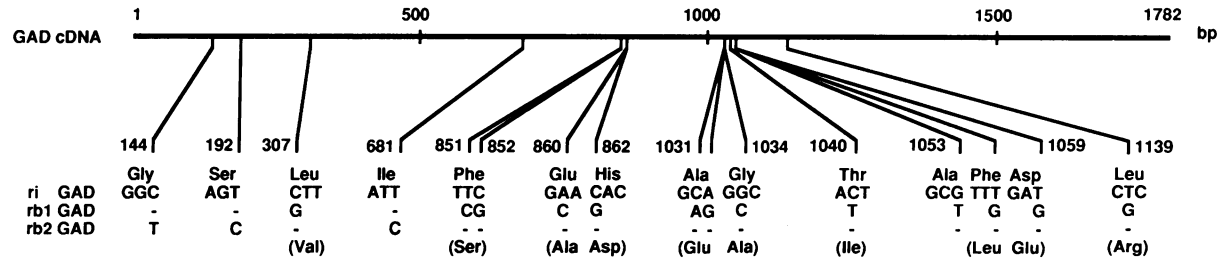

FIG. 1. Allelic variation of rat GAD67. riGAD, rat islet GAD67; rb1GAD, rat brain GAD67 (19); rb2GAD, rat brain GAD67 (30); bp, base pairs.

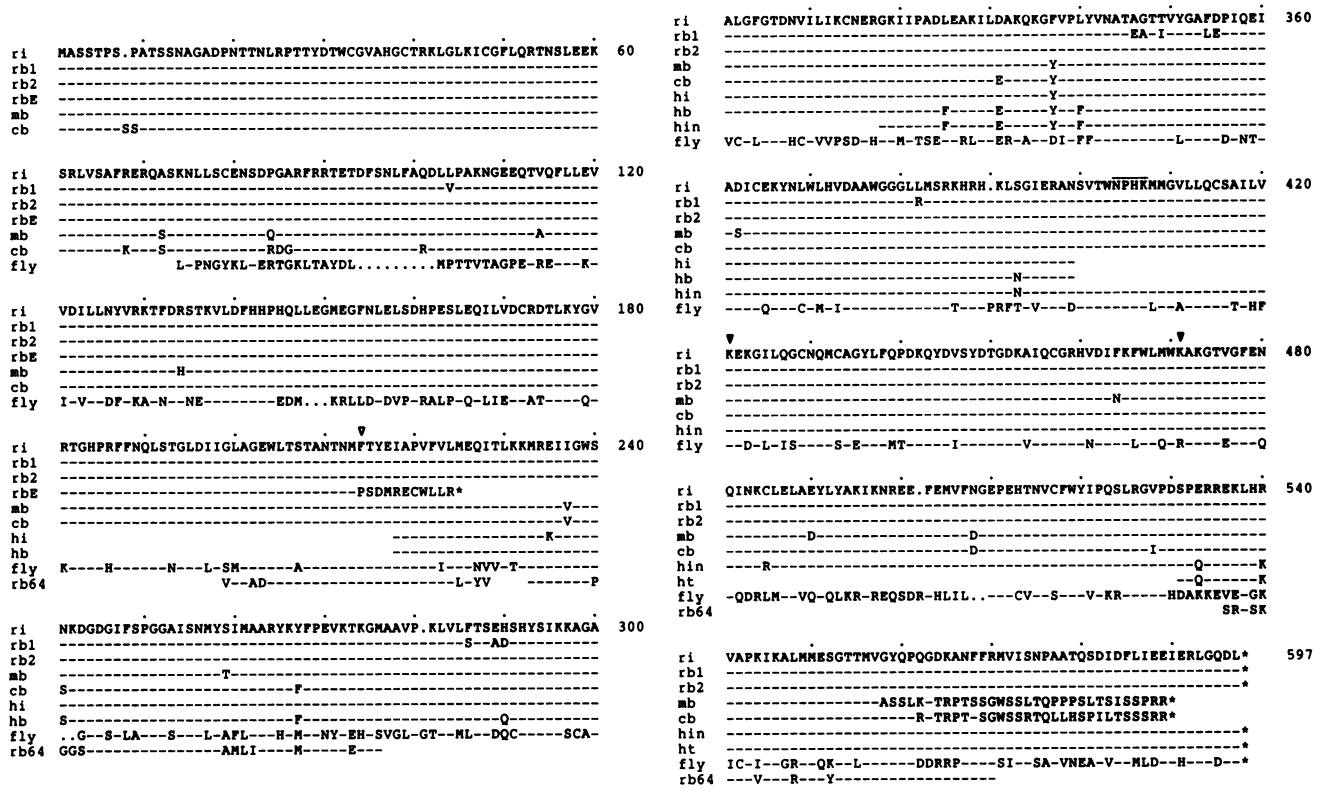

FIG. 2. Alignment of GAD sequences. ri, rat islet GAD67; rb1, rat brain GAD67 (19); rb2, rat brain GAD67 (30); rbE, rat brain embryonic peptide EP10 (33); mb, mouse brain GAD67 (31); cb, cat brain GAD67 (21); hin, human insulinoma GAD67; hi, human islet GAD67 (34); hb, human brain GAD67 (34); ht, human testes GAD67 (22); fly, *Drosophila* GAD (35); rb64, rat brain GAD64 peptides (7). Dashes, identity with rat islet GAD67 sequence; asterisks, translational stop codons; dots, gaps introduced to align sequence. Locations of introns deduced from rat brain embryonic transcript (31) (▽) and from human insulinoma GAD67 splice variant (▼) are indicated. Consensus binding site for PLP is overlined.

senting an mRNA molecule from which 50 amino acid codons had been deleted (Fig. 2). Two of nine GAD67 cDNAs sequenced had this deletion. The missing fragment may indeed represent an exon that has been deleted from the GAD67 mRNA during RNA precursor processing in the insulinoma tissue, since the DNA sequences flanking the deletion 5′-AAAAG/GGTAT . . . 140 base pairs . . . CAAAG/GGCAC-3′ are consensus splicing sites. The deletion variant was neither detected in rat islet nor in rat brain GAD67 mRNA populations in an RNase protection assay (Fig. 3).

Enzymatic Activity of Rat Islet GAD67. COS-7 cells were transiently transfected with one of two plasmid constructs containing rat islet GAD67 cDNA in either the sense or the antisense orientation within the expression vector ($pGAD_{sense}$ and $pGAD_{\alpha\text{-sense}}$). Immunostaining using the GAD antiserum S3 and the GAD peptide antiserum 1266 showed that COS-7 cells transfected with $pGAD_{sense}$ (COS_{sense}) (Fig. 4 *A–C*) but not with $pGAD_{\alpha\text{-sense}}$ ($COS_{\alpha\text{-sense}}$) (data not shown) expressed GAD immunoreactivity. The enzyme activity of the cloned rat islet GAD was demonstrated by cellular accumulation of GABA in COS_{sense} cells (Fig. 4 *D–F*) as well as by the ability of COS_{sense} extracts to catalyze the conversion of [^{14}C]glutamate to GABA as measured by $^{14}CO_2$ release (Table 1). The enzyme activity was mainly associated with the S-100 fraction, which, together with the observation that the protein segregates into the aqueous phase during detergent-phase separation, suggests a cytosolic localization of GAD67 in the COS-7 cells.

Autoantigenicity of Rat Islet GAD67. Cytosolic components from metabolically labeled rat islets, COS_{sense} cells and $COS_{\alpha\text{-sense}}$ cells were immunoprecipitated with the GAD antiserum S3, with serum from 10 control individuals, and with 10 selected 64-kDa positive sera from IDDM patients (Fig. 5). The S3 serum and all IDDM sera immunoprecipitated a large molecular mass (GAD67) as well as two smaller molecular mass (GAD64α and GAD64β) (2) polypeptides from islets. Furthermore, GAD67 in COS_{sense} cells comigrated with GAD67 in islets and was recognized by S3 and by all 10 IDDM sera (Fig. 5). This protein was not present in $COS_{\alpha\text{-sense}}$ cells. None of the control sera immunoprecipi-

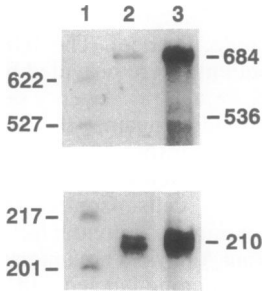

FIG. 3. RNase protection experiment. Total RNA (10 μg) from isolated rat islets or rat brain tissue was hybridized with radiolabeled GAD67 probe (100,000 dpm) and control cyclophilin probe (10,000 dpm) at 42°C overnight. Samples were treated with RNase A (1 unit/ml) and RNase T1 (200 units/ml) for 30 min at 37°C to remove single-stranded RNA, followed by digestion with proteinase K. RNA hybrids were extracted with phenol/chloroform and precipitated with ethanol. The protected RNA was analyzed on a 6% sequencing gel and visualized by autoradiography. Lanes: 1, single-stranded DNA molecular size marker; 2, probe protected by rat islet RNA; 3, probe protected by rat brain RNA. Expected migration of bands: GAD67 probe protected by normal GAD67 mRNA (684 bases); GAD67 probe protected by GAD67 human insulinoma splicing variant (536 bases); control probe protected by cyclophilin mRNA (210 bases).

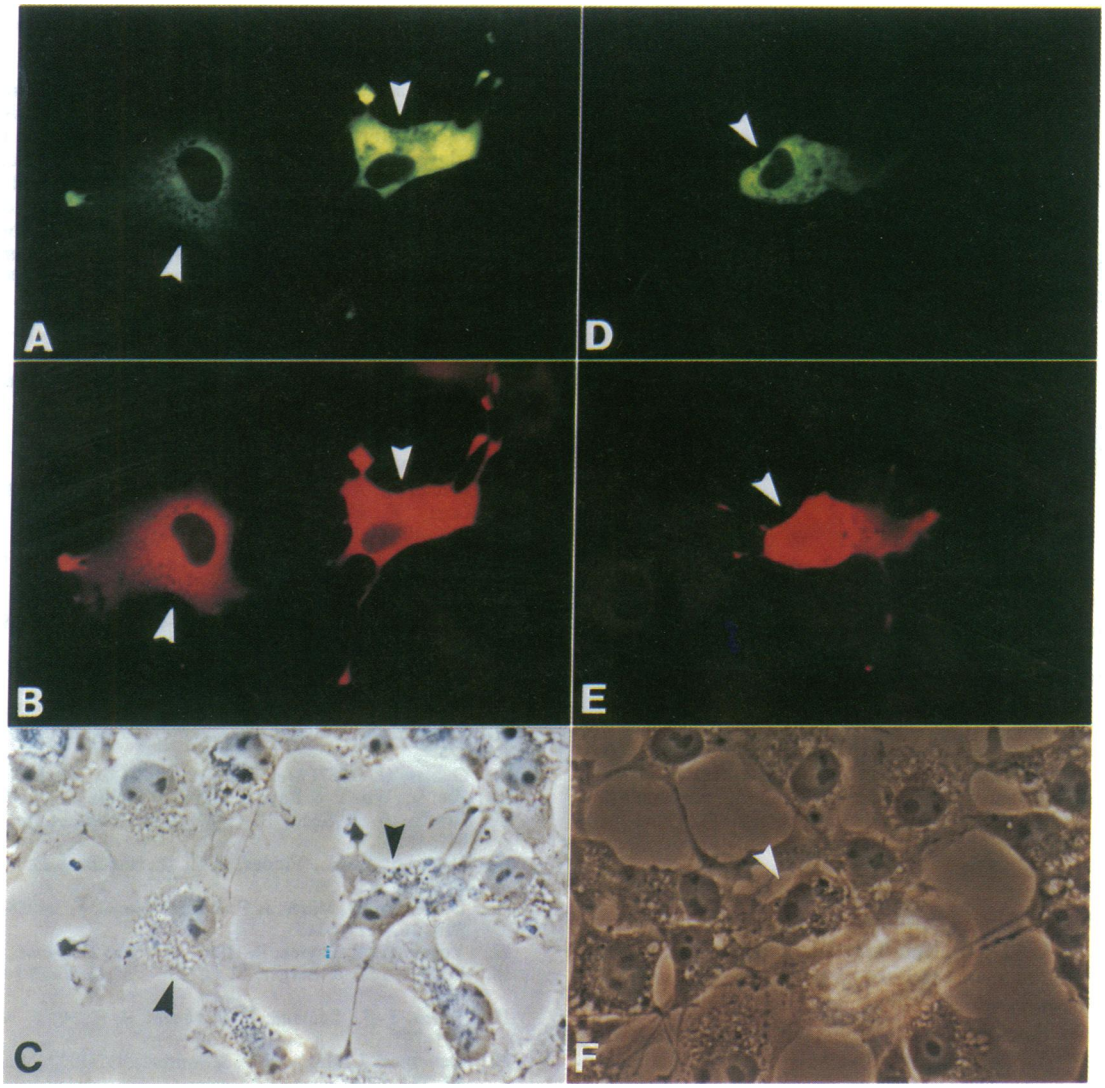

FIG. 4. Functional expression of rat islet GAD67 causes GABA accumulation in heterologous cells. COS-7 cells transiently transfected with $pGAD_{sense}$ were double stained with the GAD antiserum S3 (*A*) and the C-terminal peptide antiserum 1266 (*B*), showing that the rat islet GAD67 protein is expressed and is recognized by both antisera simultaneously (*A–C*). All GAD-positive cells (antiserum S3 in *D*) accumulated high levels of GABA immunoreactivity (anti-GABA in *E*), reflecting the enzymatic activity of the transfected rat islet GAD67 protein. Arrowheads localize positively transfected cells in the stained culture (*C* and *F*, phase contrast).

tated GAD67 from islets (Fig. 5). However, a weak signal was seen on long x-ray exposures of COS_{sense} material precipitated with control sera (Fig. 5), which also precipitated a major non-GAD 69-kDa molecule, which did not react with the GAD antiserum 1266 in immunoblotting (Fig. 5). We speculate that the presence of this protein, which is specific for the transfected cells expressing high amounts of GAD, could reflect nonspecific coprecipitation phenomena due to cellular stress.

DISCUSSION

In the present study we show that serum from IDDM patients, but not from nondiabetic individuals, contains autoantibodies that recognize GAD67 in rat islets. Rat islet GAD67 and a fragment of human insulinoma GAD67 are shown to be identical to brain GAD67. The rat islet GAD67, when transfected into COS-7 cells, is a hydrophilic protein with GAD enzyme activity, mainly localized to the cytosol fraction; it is immunoprecipitated by IDDM sera and comigrates electrophoretically with the hydrophilic GAD67 in islets. Finally, the existence of allelic variation in rat as well as in human GAD67 is demonstrated.

Table 1. GAD enzyme activities in transfected COS-7 cells

	COS_{sense}	$COS_{\alpha\text{-sense}}$
Crude extract	0.36	0.017
Cytosolic fraction	1.84	0.088
Membrane fraction	0.32	0.015

Results are expressed as milliunits/mg.

We conclude that islet and brain GAD67 are identical and are autoantigenic in IDDM. The availability of recombinant GAD67 will allow more detailed studies on the prevalence of GAD67 autoreactive antibodies and T cells in the population to identify prediabetic individuals.

The most simple explanation for the observed coprecipitation of GAD64 and GAD67 would be that the autoantibodies cross-react with common epitopes likely to reside within sequences that are well conserved between the two forms. Although only very sporadic peptide sequence information is currently available on GAD64 (7), such areas of high homology do exist (Fig. 2). However, the variable ratio between the amount of GAD67 and GAD64 precipitated by sera from different IDDM patients (Fig. 5) cannot be accounted for by autoantibodies recognizing common epitopes but implies the existence of distinct epitopes on one or both forms. It is

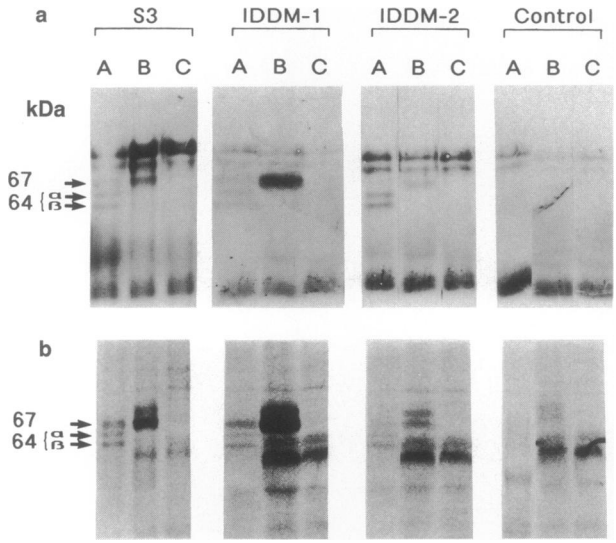

FIG. 5. Immunoprecipitation experiment using sheep anti-GAD antiserum (S3), two IDDM sera (IDDM-1 and IDDM-2), and one control serum (Control) to immunoprecipitate the various GAD components (arrows) from rat islet aqueous phase (lanes A), from COS-7 cells transfected with $pGAD_{sense}$ (lanes B), and from COS-7 cells transfected with $pGAD_{\alpha\text{-sense}}$ (lanes C). Immunoprecipitates were electrophoresed and immunoblotted. (*a*) The immunoblot was then stained with the C-terminal GAD67 peptide antiserum 1266 to identify GAD immunoreactive proteins. (*b*) Subsequently, the same blot was autoradiographed to visualize all immunoprecipitated proteins. Arrows indicate the position of GAD67, GAD64α, and GAD64β.

therefore evident that the antibody response to GAD in IDDM is polyclonal and is directed against different epitopes on GAD64 and GAD67, although these data do not exclude the possibility that cross-reactivity between the two forms may indeed occur as well.

The present data, taken together with other previously reported data, strongly suggest the existence of allelic variation in rat and human GAD67. The variation among human GAD67 sequences previously ascribed to the tissue of origin (34) thus may rather reflect allelic differences. It remains to be shown whether the 10-amino acid difference between riGAD and rb2GAD on the one hand and rb1GAD on the other has any functional, immunological, or pathogenic consequences. An interesting question is whether the genetic susceptibility to develop IDDM is associated with allelic polymorphism of the GAD67 gene.

Human insulinoma tissue was found to express an alternatively spliced GAD67 variant lacking amino acid codons 421–470 (Fig. 2). However, it is not clear whether this transcript has any physiological relevance, since it was absent in rat islets and rat brain (Fig. 3). Alternative processing of GAD67 mRNA has been reported to account for the major GAD mRNA species in embryonic rat brain (32), where it encodes a truncated, enzymatically inactive 25-kDa GAD67 peptide fragment (33) (Fig. 2). Thus, in the embryonic rat brain, differential GAD mRNA splicing is a feature of developing brain cells. Whether this is true for islet tissue as well remains to be investigated. Further investigation of differential splicing in adult human islets will clarify whether this phenomenon is specific for islet tumor cells, which might represent a lower level of islet cell differentiation (36).

Comparison of all presently available GAD sequences shows a remarkable conservation between species, (Fig. 2). Interestingly, the *Drosophila* sequence appears to resemble GAD64 and GAD67 at different positions, suggesting that mammalian GAD64 and GAD67 are derived from a presumptive common ancestral gene (37).

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