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Baculovirus-Mediated Expression of Human 65 kDa and 67 kDa Glutamic Acid Decarboxylases in SF9 Insect Cells and Their Relevance in Diagnosis of Insulin-Dependent Diabetes Mellitus[1]

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cDNAs coding for the full-length human 65 and 67 kDa glutamic acid decarboxylases (GAD_{65} and GAD_{67}) were amplified from pancreas and hippocampus cDNA libraries by polymerase chain reaction, respectively. Both cDNAs were inserted into a baculovirus vector which mediated highly efficient expression of the human GAD_{65} and GAD_{67} with histidine-hexapeptides as affinity ligands at their C-termini in *Spodoptera frugiperda* (Sf9) cells. The recombinant GAD proteins were purified to homogeneity by affinity chromatography using a metal-chelating matrix. The infected Sf9 insect cells expressed the recombinant human GAD_{65} and GAD_{67} with natural-like conformations, as confirmed by measurement of their enzyme activities as well as their fully restored autoantigenicities. Immunoprecipitation of metabolically labeled infected Sf9 cells demonstrated the autoantigenic potential of the recombinant GAD proteins. The practicability of using recombinant GAD_{65} and GAD_{67} derived from the baculovirus expression system for the development of an immunoassay for the diagnosis of insulin-dependent diabetes mellitus is discussed.

Type 1 (insulin-dependent) diabetes mellitus (IDDM) results from selective destruction of the insulin-producing β-cells of the pancreas mediated by a chronic, clinically silent, autoimmune process (*1, 2*). The detection of circulating islet cell specific autoantibodies in non-diabetic individuals indicates a risk for IDDM and has lead to a search for the β-cell specific components that may be the targets of this autoimmune response (*3, 4*). Autoantibodies directed to a 64 kDa protein seem to be the earliest detectable β-cell antibodies, being found up to 8 years before the clinical onset of IDDM in 80-90% of patients (*5-12*). The 64 kDa islet antigen representing one of the major target antigens in IDDM has been identified as glutamic acid decarboxylase (GAD). GAD is also expressed in brain tissue (*13*). Recent studies of GAD expression and purification have demonstrated at least two isoforms of GAD, with apparent molecular masses of 65 kDa (GAD_{65}) and 67 kDa (GAD_{67}), which are encoded by two genes located on two different chromosomes (*14, 15*). Both GAD_{65} and GAD_{67} are expressed in islet cells, and both forms are recognized by sera from patients with IDDM (*16-21*). Reliable measurement of these anti-GAD autoantibodies is considered to be highly predictive of the development of IDDM and coincides with the gradual loss of β-cells. Early diagnosis will provide an opportunity for treatments that may forestall or prevent serious health problems usually associated with the clinical stage of IDDM.

If GAD proves to be one of the primary antigen(s) in the pathogenesis of IDDM, the corresponding autoantibodies in the sera of patients with IDDM should be an important diagnostic marker for the clinical manifestation of IDDM or prediabetic insulitis. In order to obtain large amounts of natural and autoantigenic human GAD_{65} and GAD_{67} it was necessary to take advantage of recombinant DNA techniques combined with a suitable eukaryotic expression system. In this report we describe the isolation of cDNA clones coding for the full-length human GAD_{65} and GAD_{67}, and their high level expression as fusion proteins with histidine-hexapeptides at their C-termini in *Spodoptera frugiperda* (Sf9) cells mediated by a recombinant baculovirus. The histidine-hexapeptide fusion moiety allowed the specific purification of the recombinant GAD proteins by affinity chromatography with a metal-chelating matrix (*22*). The autoantigenicity of both recombinant human GAD_{65} and GAD_{67} was examined by immunoprecipitation using sera of patients with recent-onset IDDM.

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Abbreviations: AET, 2-aminoethylisothiouronium bromide; GAD_{65} and GAD_{67}, 65 kDa and 67 kDa glutamic acid decarboxylases; IDDM, insulin-dependent diabetes mellitus; IPTG, isopropylthiogalactoside; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLP, pyridoxal-5-phosphate; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Isolation and Characterization of GAD_{65} and GAD_{67} cDNA Clones—cDNA clones coding for the human GAD_{65} and GAD_{67} were isolated from a human pancreas cDNA library in λ-gt11 (Clontech) or a human hippocampus cDNA library in λ-ZAPII (Stratagene) by PCR amplification. Both the 5′-PCR primer (5′-TCTCTCCCGGGATC-CGAGCTGATGGCITCTTCIACICCTTC-3′) and the 3′-PCR primer (5′-TCTCTCGGTACCTCGAGTTACAGATC-CTGGICCAGTCTTTCTATC/TTCCTC-3′) were designed to add terminal restriction sites for cloning purposes and corresponded to the homologous cDNA sequence of the published rat brain 67 kDa GAD (*23*). For PCR amplification recombinant DNA was isolated from 2×10^6 plaques of the λ-cDNA libraries according to a standard protocol with a λ-phage DNA isolation kit (Quiagen). A total of 1.0 μg of recombinant cDNA was amplified with 2 units of Taq polymerase (Amersham) in a 100 μl reaction mixture comprising 10 mM Tris/HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 4 pmol of each primer, and 100 μM dNTPs. The amplification reaction was carried out for 30 cycles with the following cycling times: denaturation, 1 min at 95°C, annealing, 2 min at 60°C, and primer extension, 2 min at 72°C. Additional 3′-PCR primers were designed to add the coding sequence (CATCACCATCACCATCAC) for the histidine-hexapeptides at the 3′-ends of both isolated GAD cDNAs.

Expression of Human GAD_{65} and GAD_{67} in Sf9 Cells—The isolated cDNAs coding for either full-length human GAD_{65} or GAD_{67} were inserted into baculovirus transfer vector pVL1393 (Invitrogen) to construct cDNA clones coding for recombinant fusion proteins with GAD_{65} and GAD_{67} at their N-termini and histidine-hexapeptides at their C-termini, respectively. *Spodoptera frugiperda* (Sf9) cells were cotransfected with the recombinant transfer vectors and the linearized wild type baculovirus *Autographa californica* by lipofection at a density of 1.6×10^6 cells/ml with a multiplicity of infection (MOI) of 5 according to general baculovirus methods (*24-26*). After plaque purification, positive recombinant baculovirus clones were selected for expression according to standard protocols (*27*). The infected Sf9 cells were grown in SF900 medium (Gibco) containing 0.04% fetal calf serum as a suspension culture for 48 h prior to harvesting. Extracts of Sf9 cells infected only with the wild type virus and non-recombinant pLV1393 were used as controls (MOCK).

Purification of Recombinant Human GAD_{65} and GAD_{67}—Infected Sf9 cells were cultured as a suspension in SF900 medium (Gibco) containing 0.04% fetal calf serum for 48 h and then sedimented prior to lysis. About 8 ml of sedimented cells was resuspended in 30 ml of lysis buffer (20 mM Tris/HCl, pH 7.4, 1 mM PMSF, 1 mM AET, 0.02 mM PLP, and 2 μg/ml of the proteinase inhibitors, leupeptin, aprotinin, bestatin, and pepstatin), homogenized with 10 strokes of a glass-teflon homogenizer at 0°C, and then separated into soluble and insoluble cell fractions by centrifugation at $100,000\times g$ and 4°C for 30 min. The supernatant was adjusted to 40 mM Tris/HCl, pH 7.6, and 0.5 M NaCl, and then directly applied onto a chelating Sepharose-FF column charged with nickel ions according to the manufacturer's standard protocol (Pharmacia). The loaded column was developed with 10, 30, and 500 mM imidazole in lysis buffer containing 0.5 M NaCl. The recombinant GAD proteins eluted with 500 mM imidazole were supplemented with 40% sucrose and directly used for immunological assays.

Western Blotting Analysis of Recombinant GAD_{65} and GAD_{67}—The protein fractions were separated by 10% SDS-PAGE under reducing conditions and then transferred to nitrocellulose filters (Amersham) using a trans-blot semi-dry electrophoretic transfer cell (Bio-Rad). The unoccupied protein-binding sites on the filters were blocked with 5% nonfat drid milk in TBST-buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20). The immobilized proteins were incubated for 90 min with 500-fold dilutions of autoimmune sera. The bound antibodies were visualized with anti-human immunoglobulins conjugated with alkaline phosphatase (Promega) as previously described (*22*).

Immunoprecipitation of Metabolically Labeled GAD_{65} and GAD_{67}—Approximately 5×10^6 infected Sf9 cells per culture dish of 100 mm diameter were cultured in Grace's medium (Gibco) containing 10% fetal calf serum for 36 h at 27°C prior to adjustment of the cells to a medium free of serum and methionine for 60 min and subsequent labeling with 10 ml serum-free medium containing 200 μCi [^{35}S]-methionine for an additional 6 h at 27°C. The cells were lysed with 0.5 ml of hypotonic buffer (20 mM potassium phosphate, pH 7.0, 2 mM EDTA, 2 mM PMSF, 1 mM AET, 2 μg/ml aprotinin, 0.2 mM PLP) and then centrifuged at $100,000\times g$ for 30 min. The pellets were resuspended in 20 mM potassium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 1% Trasylol, 1% Triton X-100 for 2 h at 4°C followed by centrifugation at $33,000\times g$ for 30 min. One hundred microliter aliquots of the supernatants were mixed with 25 μl of antiserum and then incubated overnight at 4°C. The immune complexes were bound to 30 mg of protein A-Sepharose (Pharmacia) at 4°C for 2 h, washed sequentially with wash-buffer (100 mM Tris/HCl, pH 9.0, 500 mM LiCl, 1% 2-mercaptoethanol, 1% Triton X-100) and PBS, and then eluted and separated by 10% SDS-PAGE, followed by fluorography (*10*). All sera tested for immunoprecipitation had been pre-cleared with a pool of normal human sera prior to precipitation with the IDDM patient sera (*10*).

Measurement of the Enzyme Activities of Recombinant GAD_{65} and GAD_{67}—GAD activity was measured according to the protocol of Krieger and Heller (*28*). The production of [^{14}C]CO_2 on decarboxylation of 0.1 μCi L-[1-^{14}C]glutamate (Amersham) was determined in 200 μl of tissue or cell homogenates in cell-lysis buffer (50 mM KH_2PO_4, pH 7.0, 1 mM EDTA, 1 mM AET, 0.2 mM PLP, 1% Triton X-100). The GAD activity was calibrated with respect to both incubation time and protein concentration, and expressed as mU/mg total cellular protein (1 unit = 1 μmol [^{14}C]CO_2/min).

Anti-GAD Antibodies—All autoimmune sera tested in the present study were derived from recently diagnosed IDDM patients, and were assayed for anti-GAD_{65} autoantibodies by immunoprecipitation using isolated porcine pancreatic islet cells after metabolical labeling with [^{35}S]-methionine (*5, 10*). The patient serum designated as anti-GAD924 was found to be strongly reactive with linear autoantigenic epitope(s) of the human GAD_{65} (*29*), and

therefore selected for Western blotting analysis of the expressed recombinant GAD_{65}. A mouse monoclonal anti-GAD antibody specific for GAD_{67} was generated against a bacterially expressed and purified GAD_{67} peptide of 46 amino acids in length, and generously made available by Drs. B. Ziegler and M. Ziegler (Institute of Diabetes, University of Greifswald, Karlsburg, Germany). The culture supernatant of a corresponding mouse hybridoma cell line containing the monoclonal anti-GAD_{67} antibody was diluted 200-fold for Western blotting analysis.

RESULTS

Isolation and Characterization of Human GAD_{65} and GAD_{67} cDNA Clones—DNAs coding for human GAD_{65} and GAD_{67} were isolated from human pancreas and hippocampus cDNA libraries by PCR amplification based on the homologous sequence of published rat brain GAD_{65} cDNA, respectively (*23*). cDNA fragments of 1.788 and 1.819 kb in length coding for 585 and 594 amino acids of GAD_{65} and GAD_{67} were confirmed by DNA sequence analysis, respectively (Fig. 1). At the nucleotide level, the human GAD_{65} and GAD_{67} cDNAs revealed sequence homology of 64.1%, and were 62.9 and 90.1% identical to the rat brain GAD_{67} cDNA, respectively (*23*). At the amino acid level, the human GAD_{65} and GAD_{67} exhibited an overall sequence homology of 64%.

Expression of Human GAD_{65} and GAD_{67} in Sf9 Insect Cells and Their Western Blotting Analysis—Both the 1.788 kb GAD_{65} cDNA and 1.819 kb GAD_{67} cDNA were inserted into baculovirus transfer vector pVL1393 for the infection of Sf9 insect cells. The obtained constructs, pAcGAD2 and

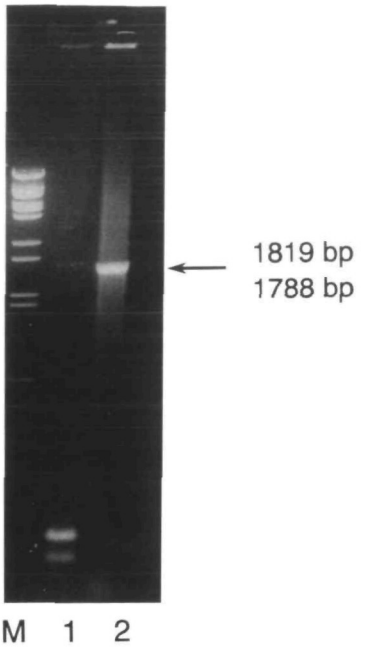

Fig. 1. **PCR amplification of cDNAs coding for human GAD_{65} and GAD_{67}.** The total input of 1.0 μg of recombinant DNA derived from human hippocampus (lane 1) and human pancreas cDNA libraries (lane 2) was amplified by PCR. Aliquots of 10 μl from the PCR reaction mixture (100 μl) were separated in an 1% agarose gel and visualized by staining with ethidium bromide. A 1,819 bp band (lane 1) and a 1,788 bp band (lane 2) representing full-length cDNAs coding for human GAD_{67} and GAD_{65} were obtained, respectively. Size markers (M).

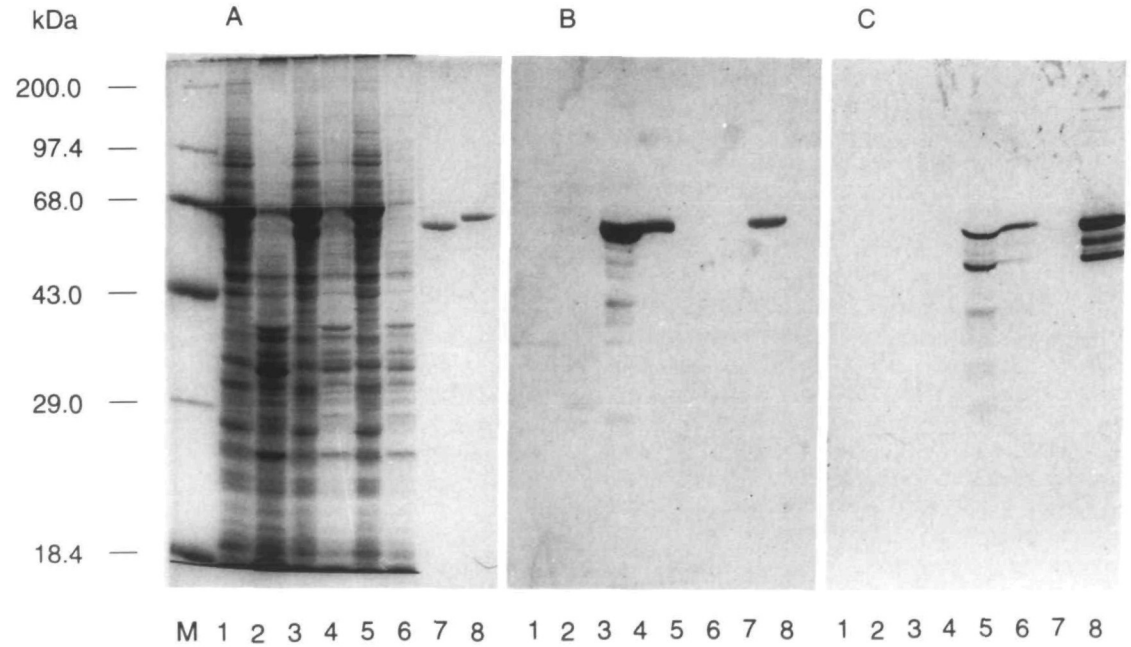

Fig. 2. **Expression and Western blotting analysis of human GAD_{65} and GAD_{67} derived from Sf9 insect cells.** Infected Sf9 cells were cultured for 48 h, and then lysed in buffer containing 0.3% Nonidet P-40 by freezing and thawing. The homogenate was separated into soluble and insoluble cell fractions by centrifugation. Aliquots representing approximately 10^5 cells were separated by 10% SDS-PAGE and stained Coomassie Brilliant Blue (A) or analyzed by Western blotting using the anti-GAD924 serum from a patient with IDDM (B) or the mouse monoclonal anti-GAD_{67} antibody (C). Size markers (M). Soluble (lane 1) and insoluble fraction of MOCK cells (lane 2), soluble (lane 3) and insoluble fraction of GAD_{65}-expressing Sf9 cells (lane 4), soluble (lane 5) and insoluble fraction of GAD_{67}-expressing Sf9 cells (lane 6), and 2.5 μg of purified GAD_{65} (lane 7) and GAD_{67} (lane 8), respectively.

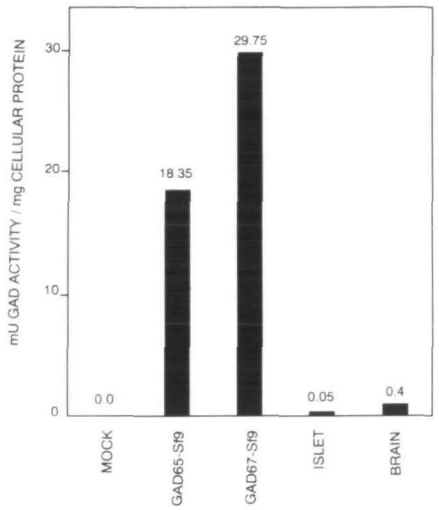

Fig. 3. **Measurement of GAD activities in infected Sf9 insect cells.** Sf9 cells infected with the wild type baculovirus (MOCK), Sf9 cells expressing GAD_{65} (GAD_{65}-Sf9) and GAD_{67} (GAD_{67}-Sf9), isolated porcine pancreatic islet cells and porcine brain were homogenized and used for the measurement of GAD activities.

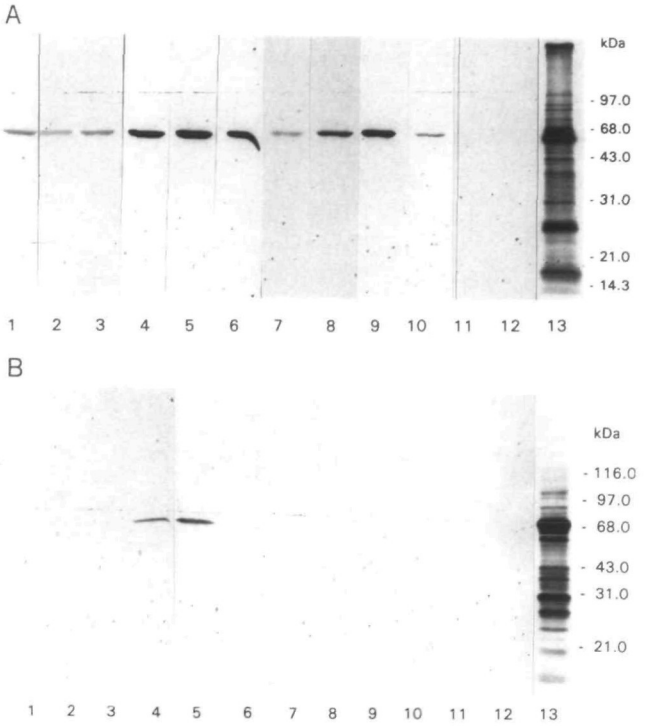

Fig. 4. **Immunoprecipitation of recombinant human GAD_{65} and GAD_{67} with IDDM patient sera.** Soluble cell fractions of metabolically labeled recombinant Sf9 cell cultures expressing human GAD_{65} (A) and GAD_{67} (B) were analyzed by immunoprecipitation using sera from patients with recently diagnosed IDDM (lanes 1-10) or with two sera from healthy individuals (lanes 11 and 12). Lysates of corresponding infected Sf9 cells were analyzed prior to immunoprecipitation (lane 13). The proteins were separated by 10% SDS-PAGE and visualized by fluorography.

pAcGAD1, directed the high-level expression of recombinant fusion proteins of human GAD_{65} and GAD_{67} with histidine-hexapeptides at their C-termini and with the expected apparent molecular masses under the control of the baculovirus polyhedrin promoter, respectively (*24*). Western blotting analysis with either a GAD_{65}-specific autoimmune serum from a patient with IDDM designated as anti-GAD924 (Fig. 2B) or with a GAD_{67}-specific mouse monoclonal anti-GAD antibody (Fig. 2C) demonstrated that infected Sf9 cells synthesized the recombinant GAD_{65} and GAD_{67} as both soluble and insoluble proteins (Fig. 2, lanes 3 and 4, and 5 and 6). No expression of any GAD isoform was detected in the control Sf9 cells (MOCK) infected only with the non-recombinant vector (Fig. 2, lanes 1 and 2). The smaller bands probably represent premature translation-termination events and/or limited degradation of the recombinant GAD proteins.

Enzyme Activities of Expressed Human GAD_{65} and GAD_{67}—The recombinant human GAD_{65} and GAD_{67} expressed by the baculovirus in Sf9 cells were examined for enzyme activity in order to determine their biological nature and conformation in comparison with natural GAD isolated from porcine islet cells and porcine brain. As shown in Fig. 3, both the infected Sf9 cell lines producing human GAD_{65} (GAD65-Sf9) and GAD_{67} (GAD67-Sf9), respectively, exhibited approximately 370 and 45-fold, and 600 and 75-fold increases in GAD enzyme activity as measured in porcine islet cells and brain, respectively. No GAD activity was detectable in control Sf9 cells (MOCK).

Purification of Recombinant Human GAD_{65} and GAD_{67}—The recombinant human GAD_{65} and GAD_{67} molecules were expressed as histidine-hexapeptide fusion proteins to ensure a simplified purification protocol. The method involves single-step affinity chromatography of the soluble cell fraction using a metal-chelating matrix charged with Ni^{2+} ions (*22*). To maintain natural-like conformation of both GAD forms, the chromatography of the recombinant proteins was modified, being carried out at physiological pH and without any detergents. The bound recombinant GAD_{65} and GAD_{67} proteins were eluted from the matrix with 0.5 M imidazole (*30*). The method allowed highly feasible and efficient isolation of recombinant GAD_{65} and GAD_{67} proteins with purities approaching homogeneity, as shown by SDS-PAGE and Western blotting analysis (Fig. 2, lanes 7 and 8). About 3-5 mg of each soluble recombinant protein, GAD_{65} and GAD_{67} was isolated from 1,000 ml of suspension culture of infected Sf9 cells.

Immunoprecipitation of Recombinant Human GAD_{65} and GAD_{67} with IDDM Patient Sera—Sera from patients with newly diagnosed IDDM who previously showed high titers of anti-GAD_{65} autoantibodies on immunoprecipitation of metabolically labeled porcine islet cells (*10*) were used to demonstrate the corresponding antigenicity of the recombinant human GAD_{65} as well as GAD_{67}. Infected Sf9 cells were radioactively labeled with [^{35}S]methionine and then immunoprecipitated with either ten sera from patients with IDDM or two sera from healthy individuals (Fig. 4). All of the selected IDDM sera precipitated the recombinant GAD_{65} (Fig. 4A, lanes 1-10). Only two (20%) of the same ten IDDM sera actually recognized additionally the recombinant GAD_{67} (Fig. 4B, lanes 4 and 5). No immunoprecipitation of either recombinant GAD protein was observed with control sera (Fig. 4, lanes 11 and 12).

DISCUSSION

Unlike other autoimmune endocrinopathies, insulin-dependent diabetes mellitus (IDDM) usually strikes the very young with many severe complications, including major morbidity and mortality (*6, 10, 31*). The future goal of diabetes type 1 research is to elucidate the molecular mechanisms involved in the early establishment and pathogenesis of IDDM. From the clinical perspective, two main goals are important. One is to obtain more detailed knowledge for the early diagnosis of the disease, and the other is to increase the knowledge on the pathology and origin of the disease, and the possibilities to improve therapeutic methods, respectively.

The diagnostic implications are of major importance for a disease such as IDDM, which appears to be a very slow process and remains asymptomatic for a long preclinical period. Several groups have reported that islet cell specific antibodies are significant markers, and have identified individuals with a high risk for IDDM (*5-11*). One of the earliest detectable islet cell autoantibodies is the anti-GAD_{65} autoantibody, which could be highly predictive for the development of IDDM (*6*). In pancreatic β-cells two GAD proteins were identified, with apparent molecular weights of 65 kDa (GAD_{65}) and 67 kDa (GAD_{67}), which are both autoantigenic (*17, 18, 20, 32*). Current immunoassays such as immunofluorescence and immunoprecipitation ones involve frozen sections of pancreas or a suspension of isolated pancreatic islet cells as antigenic substrates for the detection of autoantibodies to GAD, respectively. These assay systems depend on the wide variability of the antigen concentration in human or animal tissues, leading to inconsistent test results. Because of the limited amounts and purity of the two GAD forms, the discrimination on measurement of the autoantibodies to GAD_{65} and GAD_{67} is hardly possible. It is thus be of importance to develop an alternative and specific immunoassay system (ELISA) which involves either purified recombinant human GAD_{65} or GAD_{67} as an antigenic target in order to eliminate all disadvantages caused by natural GAD sources.

The repertoire of expression systems available for the production of foreign recombinant proteins is large, and extends to several examples of bacterial, yeast, and mammalian cell systems. The choice of the best expression system for a particular gene product is therefore sometimes difficult, and should be based on certain criteria such as the yield and biological activity of the recombinant proteins. Several approaches involving bacterial expression systems for the synthesis of recombinant human GAD_{65} and GAD_{67} as antigenic targets for immunoassays were only partly successful (*18*). The main bulk of recombinant GAD expressed in bacteria lacked the correct modifications and conformation, and was thus not recognized representatively by sera from patients with IDDM, indicating the importance of conformational antigenic epitopes within the GAD proteins (*13*). Less than 20% of the IDDM patients developed autoantibodies which were able to react with the recombinant GAD_{65} derived from *Escherichia coli* (*29*).

Alternatively, the baculovirus expression system has turned out to be a very powerful system for the production of large quantities of biologically active proteins that eliminates all problems caused by prokaryotic expression systems. The baculovirus-mediated expression in Sf9 insect cells has been demonstrated to be capable of correct posttranslational modifications (*33*). Furthermore, the system is easy to scale up as a suspension culture under serum-free conditions. In the present paper we described the isolation of full-length cDNAs coding for human GAD_{65} and GAD_{67}, and their expression as recombinant GAD fusion proteins with histidine-hexapeptide moieties at their C-termini. Based on the specific complex formation between the histidine-hexapeptide moiety and the Ni^{2+} ions of a metal-chelating matrix the recombinant proteins were specifically purified to near homogeneity from the cytosolic cell fraction by single-step affinity chromatography (*22*).

The availability of the recombinant human GAD_{65} and GAD_{67} for immunoassays for a significantly early diagnosis of IDDM appears to rely on a natural-like conformation which may correspond to their autoantigenicities. Therefore, it was necessary to demonstrate the ability of the baculovirus expression system to produce the human GAD_{65} and GAD_{67} as biologically active proteins regarding their enzymatic activities and their immunoreactivities with sera from patients with clinically defined IDDM. Both the recombinant GAD_{65} and GAD_{67} fusion proteins exhibited enzyme activities in lysates of infected Sf9 cells that were about 400 to 600 times and 45 to 75 times as great as those of extracts of porcine islet cells and porcine brain, which are normally used as natural GAD sources for current immunoassays (Fig. 3). The immunoprecipitation of recombinant GAD_{65} and GAD_{67} produced in Sf9 cells correlated very well with previously described results (*10*). All ten conventionally characterized sera from patients with recent-onset IDDM (*5, 10*) were also able to specifically immunoprecipitate the recombinant GAD_{65} fusion protein derived from infected Sf9 cells (Fig. 4A), whereas only 20% could additionally recognize the recombinant GAD_{67} (Fig. 4B). Therefore, GAD_{67} seems to be of minor importance as an autoantigenic tool for the diagnosis of IDDM (*20*).

As shown in the present paper recombinant human GAD proteins derived from baculovirus-infected Sf9 cells meet all requirements of a suitable source of antigens which may replace conventially purified GAD from human and animal tissues in order to assay anti-GAD autoantibodies for the diagnosis of prediabetic insulitis and IDDM. In conclusion, the present results demonstrated that the recombinant human GAD_{65} and GAD_{67} were produced in a natural-like conformation with fully restored enzyme activities and autoantigenicities (*20*). So far, no influence on the autoantigenicity and enzyme activity of recombinant GAD proteins was observed due to the fusion with the histidine-hexapeptide. The designed histidine-hexapeptide fusion proteins provide for the first time the possibility of the simple and efficient purification of recombinant human GAD_{65} and GAD_{67}. The availability of simple immunoassays such as ELISA with purified recombinant GAD_{65} will be of importance for the screening of large populations in order to learn more about the etiology of IDDM as well as for an early diagnosis, which will allow therapeutic intervention before most of the pancreatic β-cells are destroyed.

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