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

Ludwig Mauch, Jochen Seißler, Heinz Haubruck, Neil J. Cook, Charles C. Abney, Heike Berthold, Christiane Wirbelauer, Bodo Liedvogel, Werner A. Scherbaum, and Wolfgang Northemann

*Department of Molecular Biology and **Department of Protein Chemistry, ELIAS Entwicklungslabor, Obere Hardtstrasse 18, D-7800 Freiburg, Germany; and ***Department of Internal Medicine I, University of Ulm, D-7900 Ulm, Germany

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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 led 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 (GAD65) and 67 kDa (GAD67), which are encoded by two genes located on two different chromosomes (14, 15). Both GAD65 and GAD67 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 insulinitis. In order to obtain large amounts of natural and autoantigenic human GAD65 and GAD67, 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 GAD65 and GAD67, and their high level expression as fusion proteins with histidine-hexapeptide 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 GAD65 and GAD67 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 autoantigenicity of both recombinant human GAD65 and GAD67, derived from the baculovirus expression system for the development of an immunoassay for the diagnosis of insulin-dependent diabetes mellitus is discussed.
MATERIALS AND METHODS

Isolation and Characterization of GAD65 and GAD67 cDNA Clones—cDNA clones coding for the human GAD65 and GAD67 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′-TCTCTCCGGGATCCGAGCTGATGCCTCAGTACCAGTCGAGCCAGCTTCTCTATC-3′) and the 3′-PCR primer (5′-TCTCTCGGTACCTCGAGTTACAGTCCGTGACCAGCTTCTTCCTATC-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 (Qiagen). A total of 1 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 MgCl2, 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 (CATCACATCATCACATC) for the histidine-hexapeptides at the 3′-ends of both isolated GAD cDNAs.

Expression of Human GAD65 and GAD67 in Sf9 Cells—The isolated cDNAs coding for either full-length human GAD65 or GAD67 were inserted into baculovirus transfer vector pVL1393 (Invitrogen) to construct cDNA clones coding for recombinant fusion proteins with GAD65 and GAD67 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 pVL1393 were used as controls (MOCK).

Purification of Recombinant Human GAD65 and GAD67—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-tenon homogenizer at 0°C, and then separated into soluble and insoluble cell fractions by centrifugation at 100,000 × 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 GAD65 and GAD67—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 dry 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 GAD65 and GAD67—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 μCi [35S]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 × 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 × g for 30 min. One hundred microliter aliquots of the supernatants were mixed with 25 μl of antisera and then incubated overnight at 4°C. The immune complexes were bound to 30 μg of protein A-Sepharose (Pharmacia) at 4°C for 2 h, washed sequentially with wash-buffer (10 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 GAD65 and GAD67—GAD activity was measured according to the protocol of Krieger and Heller (28). The production of [14C]CO₂ on decarboxylation of 0.1 μCi L-[1-14C]glutamate (Amersham) was determined in 200 μl of tissue or cell homogenates in cell-lysis buffer (50 mM KH₂PO₄, pH 7.0, 1 mM EDTA, 1 mM AET, 0.2 mM PLP, 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).

Anti-GAD Antibodies—All autoimmune sera tested in the present study were derived from recently diagnosed IDDM patients, and were assayed for anti-GAD65 autoantibodies by immunoprecipitation using isolated porcine pancreatic islet cells after metabolic labeling with [35S]-methionine (5, 10). The patient serum designated as anti-GAD924 was found to be strongly reactive with linear autoantigenic epitope(s) of the human GAD65 (29), and J. Biochem.
therefore selected for Western blotting analysis of the expressed recombinant GAD. A mouse monoclonal anti-GAD antibody specific for GAD was generated against a bacterially expressed and purified GAD 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 antibody was diluted 200-fold for Western blotting analysis.

RESULTS

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

Expression of Human GAD and GAD, in Sf9 Insect Cells and Their Western Blotting Analysis—Both the 1.788 kb GAD and 1.819 kb GAD, cDNAs 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 and GAD,.. 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 and GAD, were obtained, respectively. Size markers (M).

Western blotting using the anti-GAD924 serum from a patient with IDDM (B) or the mouse monoclonal anti-GAD antibody (C). Size markers (M). Soluble (lane 1) and insoluble fraction of MOCK cells (lane 2), soluble (lane 3) and insoluble fraction of GAD-expressing Sf9 cells (lane 4), soluble (lane 5) and insoluble fraction of GAD-expressing Sf9 cells (lane 6), and 2.5 μg of purified GAD (lane 7) and GAD, (lane 8), respectively.

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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 EDDM designated as anti-GAD_924 (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 [35S]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).

**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 (GAD65-Sf9) and GAD_67 (GAD67-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.
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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-GAD65 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 (GAD65) and 67 kDa (GAD67), 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 GAD65 and GAD67 is hardly possible. It is thus be of importance to develop an alternative and specific immunoassay system (ELISA) which involves either purified recombinant human GAD65 or GAD67, 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 GAD65 and GAD67 as antigenic targets for immunoassays were only partly successful (18). The main bulk of recombinant GAD expressed in bacteria lacked the correct posttranslational 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 (19). Less than 20% of the IDDM patients developed autoantibodies which were able to react with the recombinant GAD65 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 GAD65 and GAD67, 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 Ni²⁺ 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 GAD65 and GAD67 for immunoassays for a significantly early diagnosis of IDDM appears to rely on a natural-like conformation which may correspond to their autoantigenities. Therefore, it was necessary to demonstrate the ability of the baculovirus expression system to produce the human GAD65 and GAD67 as biologically active proteins regarding their enzymatic activities and their immunoreactivities with sera from patients with clinically defined IDDM. Both the recombinant GAD65 and GAD67 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 GAD65 and GAD67, 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 GAD65 fusion protein derived from infected Sf9 cells (Fig. 4A), whereas only 20% could additionally recognize the recombinant GAD67 (Fig. 4B). Therefore, GAD67 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 conventionally 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 GAD65 and GAD67 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 GAD65 and GAD67. The availability of simple immunoassays such as ELISA with purified recombinant GAD65 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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