Detection of Pancreatic Islet 64,000 M₆, Autoantigens in Insulin-dependent Diabetes Distinct from Glutamate Decarboxylase

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Abstract

Patients with insulin-dependent diabetes (IDDM) possess antibodies to islet proteins of M₆-64,000. Potential autoantigens of this M₆ include glutamate decarboxylase (GAD) and 65 kD heat shock protein. We have detected two distinct antibody specificities in IDDM that bind 50,000 M₆ or 37,000/40,000 M₆ proteolytic fragments of 64,000 M₆ proteins. In this study, we investigated relationships of these proteolytic fragments to GAD and heat shock proteins. Polyclonal antibodies to GAD bound 50,000 M₆ fragments of islet antigen. Recombinant GAD₆₇, but not GAD₆₅, blocked binding to this antigen, suggesting that 50,000 M₆ fragments are derived from islet GAD₆₅. In contrast, GAD antibodies did not recognize 37,000/40,000 M₆ fragments, and neither GAD isoforms blocked autoantibody binding to precursors of these fragments. The 37,000/40,000 M₆ fragments, but not the 50,000 M₆ fragments, were detected after trypsin treatment of immunoprecipitates from insulinoma cells that lacked expression of major GAD isoforms. Antibodies in IDDM did not bind native or trypsinized islet heat shock proteins. Thus, IDDM patients possess antibodies to GAD, but also distinct antibodies to a 64,000 M₆ protein that is not related to known GAD isoforms or heat shock proteins. (J. Clin. Invest. 1993; 92:240–248.)

Key words: insulin-dependent diabetes • glutamate decarboxylase • autoantibodies • autoantigens • heat shock

Introduction

It is generally accepted that the specific destruction of pancreatic beta cells in insulin-dependent diabetes mellitus (IDDM) is the result of an autoimmune response against one or more components of these cells (1). Studies to characterize islet cell autoantigens associated with IDDM have identified one or more proteins of M₆-64,000 that are specifically immunoprecipitated by antibodies in sera from IDDM patients (2–4). These proteins can associate with membranes and have amphiphilic properties, partitioning into a detergent phase on phase separa-

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1. Abbreviations used in this paper: GAD, glutamate decarboxylase; hsp, heat shock protein; IDDM, insulin dependent diabetes mellitus.


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included a polyclonal sheep antiserum to purified rat brain GAD (19), a rabbit antiserum (1266) to a COOH-terminal 19-amino acid peptide of GAD$_65$ (9), gift of Dr. J. Petersen, Hagedorn Research Laboratory, Gentofte, Denmark), a rabbit antiserum (5551) to a 14-amino acid peptide (VTWNPHKMMGVPLQ) spanning the pyridoxal phosphate binding site of GAD$_65$ (gift of Prof. Å. Lernmark, University of Washington, Seattle, WA) and a rabbit antiserum to Gro EL protein of Escherichia coli (20), gift of Dr. S. Hemmingsen, Plant Biology Institute, National Research Council, Saskatoon, Canada), which cross-reacts with mammalian heat shock proteins hsp 65 and hsp 70. Normal sheep serum and normal rabbit serum (Sigma Immunochemicals, St. Louis, MO) were used as controls for these antibodies.

**Iset and isletinoma cell preparations.** Islets from 5-7-d-old neonatal Wistar rats were prepared as previously described (21). STF-10-transformed hamster insulinoma line (a gift of Dr. S. J. H. Ashcroft, University of Oxford, UK) and radiation-induced rat isletinoma cells (subclone RIN-SAH, (21), gift of Dr. Å. Lernmark, University of Washington, Seattle, WA) were maintained in tissue culture in RPMI 1640 medium containing 10% fetal calf serum. Islet and insulinoma cell proteins were radiolabeled with $[^{35}S]$methionine (Amersham International, Buckinghamshire, UK) for 5 h at 37°C as described (3).

**Heat shock treatment of pancreatic islets.** In some experiments, islets were subjected to heat shock prior to labeling with $[^{35}S]$-methionine. After an overnight period in tissue culture, batches of 2000-3000 islets were incubated at 37°C (control) or 44°C (heat shock) for 20 min in 1 ml of RPMI 1640 medium containing 20 mM Hepes (pH 7.4), 5 mM NaHCO$_3$, 2 mg/ml bovine serum albumin (labeling medium) before labeling with $[^{35}S]$methionine as described (3).

**Expression of recombinant GAD.** COS-7 fibroblast cells were transfected with cDNA for rat islet GAD$_65$ and endogenous proteins labeled with $[^{35}S]$methionine as previously described (9). BHK cells were transfected with cDNA for human GAD$_65$ and GAD$_67$ (22) and maintained as stable cell lines, 64/11 and 12/8, respectively.

**Cellular extraction and immunoprecipitation.** Radiolabeled islets (2000-4000), insulinoma cells (2.5 x $10^5$ cells) or COS cell transfec- tants were homogenized on ice by 20 passes of a motor-driven Teflon/glass homogenizer (Wheaton Instruments, Millville, NJ) in 600 µl of 0.25 M sucrose, 10 mM Hepes (pH 7.4), 10 mM benzamidine, 0.1 mM p-chloromercuribenzenesulfonic acid, and 0.5% wt/vol aprotinin. The homogenate was centrifuged at 10,000 g for 30 min at 4°C. The supernatant (S1 fraction) was collected and the pellet processed for phase separation in the detergent Triton X-114 or for trypsinization at 0.5 mg/ml trypsin (type XIII, Sigma Immunochemicals) as previously described (5, 13).

Before immunoprecipitation, Triton X-114 extracts, S1 fractions, or trypsin extracts were preclarified with 50 µl of normal human serum for 2 h at 4°C followed by binding to 100 µl of protein A Sepharose (Sigma Immunochemicals) for 45 min at 4°C. A second preclarification was performed with 50 µl of normal human serum for 18 h at 4°C and 100 µl of protein A Sepharose for 45 min. Aliquots of extract representing 5 x 10$^6$ cpm of radiolabeled protein in 50 µl were incubated with 12.5 µl of serum from 5 diabetic patients or control individuals, or 2 µl of sheep or rabbit serum containing high titers of specific antibodies, for 5 h at 4°C. In competition experiments, Triton X-114 extracts of BHK cells transfected with GAD cDNA were added to radiolabeled islet extracts before incubation with sera. Immune complexes were iso- lated on 25 µl (human sera) or 10 µl (rabbit and sheep sera) of protein A Sepharose processed for SDS-PAGE on 12% polyacrylamide gels and for autoradiography as previously described (3). In some experiments, immune complexes isolated on protein A Sepharose were incubated with 100 µl of 0.1 mg/ml trypsin in 10 mM Hepes, 150 mM NaCl for 20 min at 4°C and washed with 1 ml of water before SDS-PAGE. Where indicated, urea (8 M) was included in the sample buffer for SDS-PAGE.

**Immunoprecipitation of glutamate decarboxylase and tryptic fragments from tissue extracts.** Tissue contents of GAD, and the ability of antibodies in patients' sera to immunoprecipitate GAD enzyme activ-ity from brain, islets, or insulinoma cell extracts, were measured as previously described (10).

The ability of sera to recognize GAD immunoreactivity, or tryptic fragments of GAD, from rat brain was analyzed by Western blotting. Soluble extracts of brain containing intact GAD were prepared as described (10). Trypsin extracts of brain were prepared by homogenizing brains from two Wistar rats in 5 ml of 0.25 M sucrose, 10 mM Hepes (pH 7.4), 10 mM benzamidine, 0.1 mM p-chloromercuribenzenesulfonic acid, 5 mM phenylmethylsulfonyl fluoride and particulate material sedimented at 10,000 g for 30 min. Pellets were washed once in 3 ml of 10 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM benzami- dine, 5 mM phenylmethylsulfonyl fluoride and twice in 3 ml of 10 mM Hepes (pH 7.4), 150 mM NaCl. Soluble extracts of brain were incubated with lyophilized rat GAD antibodies for 5 min on ice and the reaction stopped by the addition of phenylmethylsulfonyl fluoride to 5 mM. Nonextracted material was removed by centrifugation at 10,000 g for 15 min.

Soluble brain extracts or trypsin extracts of brain (50 µl aliquots) were incubated with 12.5 µl of serum for 5 h at 4°C. Immune complexes were isolated on protein A Sepharose, and washed and processed for SDS-PAGE as previously described (3). Separated proteins were transferred electrophoretically to nitrocellulose (23) and nitrocellulose blots incubated with a 1:1000 dilution of rabbit antiserum to GAD peptides. Antibody binding to proteins on nitrocellulose was visualized with an alkaline phosphatase-conjugated second antibody to rabbit IgG (Sigma Immunochemicals) with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate.

**Two-dimensional gel electrophoresis.** Tissue extracts were processed for two-dimensional gel electrophoresis, with isoelectric focusing in the first dimension and SDS-PAGE in the second dimension, by the method of Hochstrasser et al. (24).

**Protein content and protein synthesis.** Protein contents of tissue homogenates were determined by the Biuret reaction (25). $[^{35}S]$-methionine incorporation into proteins was determined by spotting 5-µl aliquots of tissue homogenates onto filter papers and precipitating proteins in ice-cold 10% trichloroacetic acid. Filter papers were washed extensively in 10% trichloroacetic acid and subsequently in ethanol, dried, and radioactivity incorporated into precipitated material determined by scintillation counting.

**Results.**

**Binding of antibodies to islet and brain antigens.** During our studies on autoantibody binding to proteins in detergent extracts of islets, we observed that some IDDM patients who lacked antibodies to GAD were still able to immunoprecipitate proteins of Mr, 64,000 (Fig. 1a, lane 1). These proteins were resolved as a diffuse doublet on SDS-PAGE and appeared distinct from the dominant 64,000 Mr protein immunoprecipitated by sera containing GAD antibodies (Fig. 1a, lane 2). Analysis of antibody binding to tryptic fragments of islet antigen indicated that these GAD-antibody–negative patients lacked antibodies to 50,000 Mr fragments but possessed antibodies that recognized 37,000 and 40,000 Mr fragments (Fig. 1b, lane 1). Sera from patients positive for GAD antibodies immunoprecipitated 50,000 Mr, fragments from trypsin extracts of islets (Fig. 1b, lane 2). These observations led us to investigate whether IDDM is associated with antibodies to 64,000 Mr, proteins that are distinct from GAD.

We determined whether the different fragments of islet antigen were recognized by a polyclonal sheep antiserum to rat brain GAD. Fig. 2a illustrates polypeptides immunoprecipitated from trypsin extracts of islets by antibodies in diabetic patients’ sera or by the antiserum to GAD. Diabetic patients were identified with antibodies to the 50,000 Mr, fragment of
To further characterize relationships of the antigenic fragments to GAD, patients’ sera were tested for their ability to immunoprecipitate GAD immunoreactivity from soluble or trypsin-treated rat brain extracts. Immunoprecipitated GAD polypeptides were visualized by Western blotting using an antibody raised to a COOH-terminal peptide of GAD65, which cross-reacts with GAD65 (9). Serum from diabetic patients positive for antibodies to islet 50,000, M, tryptic fragments also immunoprecipitated 64,000, M, proteins with GAD immunoreactivity from soluble brain extracts (Fig. 2 b, lanes 1 and 3), and 50,000, M, tryptic fragments from trypsin extracts of brain (Fig. 2 c, lanes 1 and 3). Sera containing only 37 k antibodies did not immunoprecipitate GAD immunoreactivity from soluble or trypsin extracts of brain (Fig. 2 b, lane 2; Fig. 2 c, lane 2). Identical results were obtained in Western blotting experiments using an antibody raised to a peptide spanning the pyridoxal phosphate binding site of GAD65 (data not shown). Thus, trypsin extracts of brain contain 50,000, M, fragments that are immunoprecipitated by antibodies in diabetic patients’ sera and that bear determinants for antibodies raised to two distinct GAD peptides. Trypsinization of brain does not generate 40,000, and 37,000, M, fragments recognized by these GAD antibodies.

The same sera were characterized for their ability to immunoprecipitate GAD67, or trypsin-treated GAD67, from extracts of COS fibroblasts transfected with rat islet GAD67 cDNA. Only one of the diabetic patients immunoprecipitated a 67,000, M, protein from Triton X-114 extracts of COS cell transfectants (Fig. 2 d, lane 1) that comigrated with a 67,000, M, protein immunoprecipitated by the sheep antiserum to GAD (Fig. 2 d lane 5). The 37k-antibody–positive sera did not immunoprecipitate GAD67 (Fig. 2 d, lanes 2 and 3).

antigen (Fig. 2 a, lane 1), antibodies to 37,000 and 40,000, M, fragments of antigen (Fig. 2 a, lane 2) and antibodies to all three fragments (Fig. 2 a, lane 3). These fragments were not recognized by control sera (Fig. 2 a, lane 4). The antiserum to GAD immunoprecipitated only 50,000, M, fragments and failed to recognize either 40,000 or 37,000, M, fragments from the islet trypsin extract (Fig. 2 a, lane 5).

Figure 1. Autoantibody recognition of islet antigens. Sera from a GAD-antibody-negative IDDM patient (lanes 1), a GAD-antibody-positive patient (lanes 2) and a control subject (lanes 3) were used to immunoprecipitate proteins from detergent extracts (a) or trypsin extracts (b) of rat islets. Polypeptides immunoprecipitated were detected by SDS-PAGE and autoradiography. The M$_{s}$ of major polypeptides recognized by antibodies are marked.

Figure 2. Immunoprecipitation of antigens in IDDM. Sera from IDDM patients (lanes 1–3), a healthy control subject (lanes 4), a sheep antiserum to rat brain GAD (lanes 5) or normal sheep serum (lanes 6) were used to detect antigens in various cell extracts. Sera were incubated with trypsin extracts [35S]methionine-labeled rat islets (a), cytosolic (S1) fractions of brain (b), trypsin extracts of brain (c), detergent extracts of COS cells transfected with cDNA for GAD$_{65}$ (d), and trypsin extracts of COS cells transfected with GAD$_{67}$ (e). Immune complexes were isolated on protein A Sepharose and separated by SDS-PAGE. In a, d and e, immunoprecipitated polypeptides were identified by autoradiography; in b and c by Western blotting with an antibody to a COOH-terminal peptide of GAD. The M$_{s}$ (×10$^{-3}$) of major polypeptides recognized by antibodies are marked. The broad band in b and c (Ig) is immunoglobulin heavy chain that binds the alkaline phosphatase–conjugated second antibody.

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analysis of antibodies in 16 recent onset IDDM patients, 9 (62.5%) had antibodies that immunoprecipitated recombinant GAD$_{67}$. No significant correlation was observed between the ability to immunoprecipitate GAD$_{67}$ from COS cell transfectants and GAD enzyme activity from rat brain extracts ($r = 0.477$, $P > 0.05$; Spearman correlation) or 37,000 $M_r$ trypsin fragments from islet extracts ($r = 0.013$, $P > 0.5$). Diabetic patients positive for antibodies to GAD$_{67}$, and the sheep antisera to GAD, also immunoprecipitated polypeptides of $\sim 57,000 M_r$ from trypsin extracts of the COS cell transfectants (Fig. 2 e, lanes J and 5).

Studies were performed to determine whether recombinant human GAD$_{65}$ or GAD$_{67}$ can compete for binding with autoantibodies to rat islet antigens. Triton X-114 extracts of BHK cells containing the recombinant proteins were added to detergent phase extracts of $[^{35}S]$methionine-labeled islets before immunoprecipitation with diabetic patient's serum. Immunoprecipitates were treated with trypsin before gel electrophoresis to distinguish antibody binding to 50,000 $M_r$, fragment-related and 37,000/40,000 $M_r$, fragment-related antigens. Addition of GAD$_{65}$, but not GAD$_{67}$, blocked binding of antibodies to proteins generating 50,000 $M_r$, fragments on trypsinization (Fig. 3). GAD$_{67}$ was ineffective even with sera strongly positive for antibodies to recombinant GAD$_{67}$ (Fig. 3, patient 2). Addition of the recombinant proteins had no effect on antibody binding to protein precursors of 40,000 and 37,000 $M_r$, fragments.

GAD and autoantigen expression in insulinoma cells. Analysis of antigen binding to antibodies in diabetic patients was extended to two transformed insulin-secreting cell lines, HIT-T15 and RIN-5AH. GAD enzyme activity was detected in homogenates of both of these cell lines, at activities of 2.28±0.28 $\mu$mol/16 h per g protein ($n = 3$) for HIT-T15 cells and 3.04±0.53 $\mu$mol/16 h per g protein ($n = 3$) for RIN-5AH cells. In parallel experiments GAD enzyme activities in rat brain and neonatal rat islets were 104.26 and 9.52 $\mu$mol/16 h per g protein, respectively (10). Sera from three diabetic patients determined to be strongly positive for antibodies to 50,000 $M_r$, fragments of antigen immunoprecipitated GAD enzyme activity from islet and brain extracts (Fig. 4). In contrast, the GAD activities in HIT-T15 and RIN-5AH cell extracts, adjusted to similar enzyme concentrations as those in islet and brain preparations, were not recognized by antibodies in the diabetic patients' sera (Fig. 4). In Western blot studies, antibodies to GAD peptides did not recognize 64,000 or 67,000 $M_r$, polypeptides in extracts of RIN-5AH or HIT-T15 cells (data not shown). Thus, GAD enzyme activity can be detected in insulinoma cell lines, but this activity is immunochemically distinct from the major isoforms of GAD expressed in brain and islets.

The ability of antibodies to recognize polypeptides in detergent extracts of radiolabeled insulinoma cells was analyzed. Antibodies in sera from 37k-antibody--positive IDDM patients specifically immunoprecipitated a 64,000 $M_r$ protein from detergent phase purified extracts of RIN-5AH cells, detectable only when immune complexes were solubilized in the presence of 8 M urea (Fig. 5). Trypsinization of the immunoprecipitated protein before electrophoresis generated 37,000 and 40,000 $M_r$, fragments, but not 50,000 $M_r$, fragments (Fig. 5). Similar results were obtained with HIT-T15 cells.

Competition experiments were performed to determine

![Figure 3](http://www.jci.org)

**Figure 3.** Blocking of antibody binding to islet antigens with recombinant GAD. Triton X-114 detergent phase extracts of $[^{35}S]$methionine-labeled rat islets were incubated with IDDM patients' sera in the presence of Triton X-114 extracts from (a) untransfected BHK cells (extract from 3.3 x 10$^5$ cells) (b) BHK cell line (64/11) transfected with cDNA for GAD$_{65}$ (3.3 x 10$^4$ cells containing 7.2 nmol/2 h GAD), or (c) BHK cell line (87/11) transfected with cDNA for GAD$_{67}$ (2 x 10$^4$ cells containing 2.0 nmol/2 h GAD). Immune complexes were isolated on protein A Sepharose and treated with trypsin (0.1 mg/ml) before SDS-PAGE. All patients were positive for antibodies to GAD from rat brain; patient 2 was strongly positive for antibodies to recombinant GAD$_{67}$ (patient 1 in Fig. 2).

![Figure 4](http://www.jci.org)

**Figure 4.** Immunoprecipitation of GAD activity from islets, brain, and insulinoma cell extracts. Nonidet P40 detergent-solubilized extracts (50 $\mu$l aliquots) of rat islets, rat brain, RIN-5AH cells, or HIT-T15 cells were incubated with sera from IDDM patients (first four bars for each tissue) and a normal control serum (fifth bar). Patients represented by bars 1-3 were positive for 50k antibodies and those represented by bars 2-4 were positive for 37k antibodies. The mean ($\pm$SEM) GAD activities in tissue extracts were 1.06±0.22 (islets), 2.19±0.37 (brain), 0.68±0.25 (RIN cells) and 3.58±0.40 (HIT cells) nmol/16 h per 50 $\mu$l ($n = 3$). GAD activity in immunoprecipitates for each serum is shown. The results are mean±SEM of 3 independent observations.

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whether insulinoma cell antigen can compete for autoantibody binding to fragments of islet antigen. Addition of trypsin extracts of unlabeled HIT-T15 cells blocked immunoprecipitation of 37,000 and 40,000 Mr fragments from [35S]methionine-labeled islet extracts but did not affect binding to 50,000 Mr fragments. The insulinoma cells, therefore, contain proteins with similar antigenic properties to 37,000 Mr and 40,000 Mr islet fragments, but may lack proteins from which 50,000 Mr fragments are derived.

**Binding of antibodies to islet proteins after heat shock treatment.** To investigate possible relationships of islet 64,000 Mr autoantigens to heat shock proteins, antibody reactivity to intact and trypsin-treated islet antigens was investigated in islets exposed to heat shock. Islets were incubated at 37°C (control) or 44°C (heat shock) for 20 min before labeling islet proteins with [35S]methionine. Incorporation of [35S]methionine into trichloroacetic acid-precipitable protein in heat shock treated islets was 93.7±2.96% (mean±SEM, n = 4) of the incorporation at 37°C. Two-dimensional gel electrophoresis of islet proteins revealed considerable increases in the intensities of proteins of Mr, 110,000, 90,000, 70,000, and 62,000 after heat shock (Fig. 6). Incorporation of label into most other individual proteins was similar under control and heat shock conditions.

The greater proportion of proteins stimulated by heat shock were recovered in the supernatant (S1) fraction after centrifugation of islet homogenates. Immunoprecipitation experiments with S1 fractions were performed to determine the ability of antibodies to recognize proteins induced by heat shock. A polyclonal rabbit antibody to Gro EL protein, the *E. coli* homologue of hsp 65, immunoprecipitated 62,000 Mr and 70,000 Mr islet proteins stimulated by heat shock treatment (Fig. 7). The 62,000 Mr hsp was not immunoprecipitated by antibodies in serum from a recent onset IDDM patient; similar results were obtained with five other IDDM patients. In experiments with extracts from heat shock–treated islets, 70,000 Mr hsp was associated with immunoprecipitates with all control and diabetic sera tested. Similar nonspecific binding of hsp 70 to immune complexes has been observed in other immunoprecipitation studies (26). A 67,000 Mr protein was immunoprecipitated from S1 fractions of islets incubated at 37°C by antibodies in 3 of the 6 IDDM patients’ sera tested, but not from extracts of heat shock–treated islets (Fig. 7).

Detergent phase fractions from islets preincubated at 37°C or 44°C were used in immunoprecipitation experiments to determine the effect of heat shock on islet antigen expression. The 64,000 Mr, antigen migrated at a slightly higher Mr than the 62,000 Mr hsp (Fig. 7). The recovery of radiolabeled 64,000 Mr antigen was reduced in experiments with detergent phase extracts of islets preincubated at 44°C.

Trypsin solubilized extracts of islet particulate proteins were used to compare expression of precursors of antigenic

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**Figure 5.** Immunoprecipitation of antigens from RIN-5AH insulinoma cells. Triton X-114 detergent phase extracts of RIN-5AH cells were incubated with serum from IDDM patients (lanes 1–5) or control subjects (lanes 6 and 7). Immune complexes were isolated on protein A Sepharose and either eluted in SDS sample buffer containing 8 M urea (left panel) or treated with trypsin (0.1 mg/ml) before elution in sample buffer (right panel). Polypeptides were separated by SDS-PAGE and detected by autoradiography. The Mr (×10⁻³) of polypeptides immunoprecipitated by antibodies in IDDM patient’s serum are marked.
Figure 6. Islet protein expression after heat shock. Islets preincubated for 20 min at 37°C or 44°C were labeled with [\(^{35}\)S]methionine for 5 h. Proteins in cytosolic (S1) fractions were separated by isoelectric focusing (IEF, first dimension) and SDS-PAGE (second dimension) and radiolabeled proteins detected by autoradiography. The \(M_s \times 10^{-3}\) of major proteins where \([^{35}\)S]methionine incorporation was stimulated by heat shock is indicated.

Tryptic fragments after heat shock. The recovery of immunoprecipitable 50,000 \(M_r\) fragment of antigen was reduced after heat shock, whereas recovery of 37,000 \(M_r\), tryptic fragment was relatively unchanged (Fig. 7). Densitometric scanning of bands on autoradiograms representing immunoprecipitated polypeptides indicated a reduction in 50,000 \(M_r\) fragment after

Figure 7. Effect of heat shock on islet antigen expression. Cytosolic (S1) fractions, detergent phase fractions or trypsin extracts of islets incubated at 37°C or 44°C before labeling with [\(^{35}\)S]methionine were incubated with rabbit antibody to Gro EL protein (lanes a), serum from a healthy control individual (lanes b) and serum from an IDDM patient (lanes c). Immunoprecipitated proteins were identified by SDS-PAGE and autoradiography. The \(M_s \times 10^{-3}\) of major proteins stimulated by heat shock are marked on left of figure and tryptic polypeptides recognized by antibodies in IDDM on right of figure. The arrowheads indicate the 67,000 \(M_r\) protein in S1 fraction, and 64,000 \(M_r\) protein in detergent phase fraction, immunoprecipitated by antibodies in IDDM.
heat shock to 9.59±1.50% (mean±SEM) of that under control conditions, whereas the density of 37,000 Mr bands was 89.9±10.7% of control conditions (n=4; P<0.01, paired t test).

**Discussion**

We have previously shown that mild trypsin digestion of islet proteins immunoprecipitated by antibodies in IDDM generates three major fragments of Mr 50,000, 40,000, and 37,000 (13). By analyzing antibody reactivity to these fragments, we have detected antibodies in sera from IDDM patients that recognize 50,000 Mr fragments and distinct antibodies that recognize common determinants on the 40,000 and 37,000 Mr fragments. We have speculated that these fragments are derived from more than one 64,000 Mr protein (13). GAD has been identified as a major 64,000 Mr antigen (6); other authors have suggested that heat shock proteins might be antigens in IDDM (14–16). In this study, we have determined whether these fragments are indeed derived from distinct proteins and have investigated possible relationships between the fragments of islet antigen, GAD, and heat shock proteins.

There is strong evidence that the 50,000 Mr component is derived from GAD. We have previously observed a high degree of correlation between the ability of antibodies to immunoprecipitate a GAD enzyme activity from rat brain extracts and the ability of antibodies to bind 50,000 Mr trypsin fragments of islet antigen (10). In this study, a polyclonal sheep antiserum raised to brain GAD immunoprecipitated 50,000 Mr fragments that comigrated on SDS-PAGE with 50,000 Mr fragments recognized by antibodies in IDDM. Furthermore, sera containing antibodies to 50,000 Mr fragments of islet antigen also immunoprecipitated fragments of identical size from trypsin extracts of brain that were recognized by antibodies to GAD peptides. In a study by Baekkeskov et al. (6), trypsin treatment was shown to generate 55,000 Mr immunoreactive fragments from both islet and brain GAD, and these 55,000 Mr polypeptides may be equivalent to the 50,000 Mr fragments observed in our study. These data suggest that the 50,000 Mr component of antigen is a trypsin polypeptide of GAD that includes the COOH-terminal domain of the enzyme. Addition of recombinant GAD65 blocked binding of antibodies to proteins, generating 50,000 Mr fragments on trypsinization. Addition of recombinant GAD65 did not affect binding, even with sera shown to be strongly positive for antibodies to GAD65. The results suggest that the 50,000 Mr fragment is derived from the lower Mr isoform of GAD, GAD65.

The 37,000 and 40,000 Mr fragments appear to be derived from a different protein to that generating 50,000 Mr fragments, since we found differences in the expression of the precursors to these fragments in islets and insulinoma cells. Thus, precursors of 37,000 and 40,000 Mr fragments were clearly present in extracts of insulinoma cell lines that lacked precursors of 50,000 Mr fragments, and in normal islets heat shock treatment reduced synthesis of 50,000 Mr fragments by more than 90%, but had little effect on synthesis of 37,000 and 40,000 Mr fragments. The results therefore support our suggestion that there is more than one 64,000 Mr autoantigen in IDDM.

Since GAD67 has also been reported to bind antibodies in IDDM (8, 9), we investigated whether the 37,000 and 40,000 Mr fragments are derived from this second GAD isoform. Our findings suggest that GAD65 is not a target for 37k antibodies. The 37,000 and 40,000 Mr fragments were not recognized by the sheep antiserum to GAD, indicating either that they are derived from a non-GAD protein or that trypsin cleavage destroys determinants recognized by this polyclonal antibody. Neither recombinant GAD65 nor GAD2 competed for autoantibody binding to precursors of 37,000 and 40,000 Mr fragments. No correlation was observed between the ability of antibodies to recognize recombinant GAD67 and the 37,000/40,000 Mr trypsin fragments and trypsinization of recombinant GAD67 generated 57,000 Mr polypeptides and not 37,000 and 40,000 Mr fragments. Finally, the RIN insulinoma cell line has been shown to lack expression of both high and low molecular weight isoforms of GAD (27), and in our experiments neither GAD immunoreactivity nor GAD mRNA were detectable in HIT or RIN cells by Western blotting with anti-GAD peptide antibodies or by Northern blotting with GAD cDNA probes (data not shown). A GAD enzyme activity was detected in insulinoma cell lines, but this GAD was not recognized by antibodies in IDDM. Despite the failure to detect GAD65 or GAD67 in RIN and HIT cells, precursors to 37,000 and 40,000 Mr antigens were clearly present in these cells. Together, these results provide strong evidence that the 37,000 and 40,000 Mr fragments are derived from proteins distinct from both GAD65 and GAD67.

The precursors of the 37,000 and 40,000 Mr antigen fragments do appear to be proteins of Mr 64,000. Diffuse bands at this Mr were immunoprecipitated from islet extracts by GAD-antibody−negative, 37k-antibody−positive sera. We, and others, have consistently failed to detect proteins of Mr other than 64,000 specifically immunoprecipitated by IDDM sera (28). Two-dimensional electrophoresis of the eluted immune complexes resolves 64,000 Mr proteins as multiple spots; two components (α and β) display charge heterogeneity and have identical mobility on gels to α and β components of GAD65 (6, 28). A third protein, designated γ, with identical Mr to the β component, but more negatively charged, has also been reported (29). Analysis of the relationship of the γ-component to 37,000 and 40,000 Mr fragments requires specific antibodies to the protein, which are currently not available.

Diffuse bands of Mr 64,000 were also immunoprecipitated from extracts of RIN cells. However, these bands were detected only when immune complexes were solubilized in the presence of urea, and the bands were weaker than the 37,000 and 40,000 Mr fragments observed on digestion of immune complexes with trypsin before electrophoresis (Fig. 5). We have previously observed that only weak bands could be detected by SDS-PAGE of immunoprecipitates from detergent extracts of islets with some sera strongly positive for 37k antibodies, and have suggested that 37k antibodies may be directed to determinants that are hidden on the detergent solubilized protein (13). However, the results of the present study show that the native antigen is indeed recognized by 37k antibodies, since precursors of the 37,000/40,000 Mr fragments were clearly present in immunoprecipitates of detergent extracts from RIN cells. The difficulty in detecting nonproteolyzed antigen appears to be related to poor solubilization of immune complexes for gel electrophoresis, rather than poor antibody recognition. Urea, a strong denaturant, improved solubilization but bands were weak compared with those seen after proteolysis. Trypsin treatment may remove hydrophobic domains that might impair solubilization or may assist in dispersing protein aggregates.
Another potential target for antibodies in diabetes is hsp 65. However, consistent with an earlier report (17), the islet 64,000 M, proteins immunoprecipitated by antibodies in diabetes migrated at a slightly higher M, than a 62,000 M, islet heat shock protein, and antibodies in sera from diabetic patients did not specifically recognize heat shock proteins in extracts of islets. Furthermore, the 37,000:40,000 M, proteins do not appear to be related to heat shock proteins, since [35S]-methionine incorporation into 37,000 M, antigenic fragments was not stimulated by heat shock. Polyclonal antibodies to hsp 65 did not cross-react with 37,000 M, fragments. These observations provide further evidence against a role for islet heat shock proteins as targets for autoantibodies in human IDDM, either in native form or after proteolysis. The results do not exclude, however, an association of IDDM with cellular immunity to heat shock proteins or that autoimmunity to islet antigens might arise through cross-reactivity to epitopes on foreign heat shock proteins.

With the exception of heat shock proteins, the synthesis of most islet cell proteins was little affected by heat shock. However, heat shock treatment caused a dramatic decrease in the expression of the GAD component of antigen. This finding, together with the failure of insulinoma cell lines to express the GAD antigen, indicates that islet GAD has an exquisite sensitivity to downregulation by a number of factors. Downregulation of GAD expression in vivo might have important consequences for maintenance of immunological tolerance to the protein. A reduced expression in early life of proteins expressed specifically by pancreatic beta cells has been shown to result in failure to induce immunological self-tolerance (30). Downregulation of GAD during early development may result in impaired tolerance to the protein and increased susceptibility to autoimmune responses. Further studies on the regulation of GAD expression in pancreatic beta cells may provide important information relevant to the initiation of autoimmune responses to the protein in IDDM.

The identification of GAD65 as an autoantigen in IDDM is a major advance toward our understanding of autoimmunity to beta cells in the disease. However, the results of this study suggest that there is more than one 64,000 M, protein autoantigen in IDDM and antibodies to 37,000 and 40,000 M, fragments are directed to a protein that is distinct from known GAD isoforms. The importance of this protein in IDDM is emphasized by studies in identical twins, where antibodies to 37,000 and 40,000 M, fragments were found to be more closely associated with diabetes development than antibodies to GAD (31). Detection of antibodies to this protein may therefore be particularly valuable in identifying individuals at risk for disease. Further characterization of the protein precursor to 37,000 and 40,000 M, fragments and its relationship to GAD are critical to our understanding of the respective roles of these antigens in the immunopathogenesis of IDDM.

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