

# Detection of Pancreatic Islet 64,000 $M_r$ 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  $M_r$  64,000. Potential autoantigens of this  $M_r$  include glutamate decarboxylase (GAD) and 65 kD heat shock protein. We have detected two distinct antibody specificities in IDDM that bind 50,000  $M_r$  or 37,000/40,000  $M_r$  proteolytic fragments of 64,000  $M_r$  proteins. In this study, we investigated relationships of these proteolytic fragments to GAD and heat shock proteins. Polyclonal antibodies to GAD bound 50,000  $M_r$  fragments of islet antigen. Recombinant GAD<sub>65</sub>, but not GAD<sub>67</sub>, blocked binding to this antigen, suggesting that 50,000  $M_r$  fragments are derived from islet GAD<sub>65</sub>. In contrast, GAD antibodies did not recognize 37,000/40,000  $M_r$  fragments, and neither GAD isoforms blocked autoantibody binding to precursors of these fragments. The 37,000/40,000  $M_r$  fragments, but not the 50,000  $M_r$  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  $M_r$  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)<sup>1</sup> 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  $M_r$  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-

tion in Triton X-114 (5). The  $\gamma$ -aminobutyric acid-synthesizing enzyme, glutamic acid decarboxylase (GAD) has been identified as a major 64,000  $M_r$  autoantigen in IDDM (6). GAD exists as multiple isoforms with different tissue-specific patterns of expression, membrane association, and reactivity with antibodies in diabetes (7–10). Two isoforms of GAD (GAD<sub>65</sub> and GAD<sub>67</sub>) have been cloned and sequenced (9, 11), and both isoforms are expressed in brain and islets (7, 8). GAD<sub>65</sub> has similar membrane-binding properties to islet antigen (8), and recombinant GAD<sub>65</sub> has been shown to compete for autoantibody binding to islet 64,000  $M_r$  protein, suggesting that the lower molecular weight isoform is related to the islet antigen (12).

By studying antibody binding to proteolytic fragments, we have identified two distinct antibody specificities to 64,000  $M_r$  proteins in sera from IDDM patients (13). Partial tryptic proteolysis of antigens immunoprecipitated from detergent extracts of islets generates three major fragments of  $M_r$  50,000, 40,000, and 37,000. Diabetic patients possess antibodies to 50,000  $M_r$  fragments, and distinct antibodies that recognize both 40,000  $M_r$  and 37,000  $M_r$  fragments of antigen. Antibodies that bind the 50,000  $M_r$  fragment (50k antibodies) also immunoprecipitate GAD from islet and brain extracts, whereas antibodies that bind 37,000  $M_r$  and 40,000  $M_r$  fragments (37k antibodies) do not recognize GAD (10). These 37k antibodies may recognize hidden determinants on GAD that are only exposed on trypsin cleavage, or may be directed to a protein distinct from GAD (10).

We have previously suggested that the 50k- and 37k-antibody specificities recognize determinants on different 64,000  $M_r$  proteins (13). Alternative candidate antigens in IDDM include a 65-kD heat shock protein (hsp 65). Both antibody and T cell reactivity to heat shock protein (hsp) 65 have been detected in the nonobese diabetic mouse model of IDDM (14, 15) and hsp 65 has been suggested to be a target for autoantibodies in human IDDM (16). However, analysis of binding of antibodies in IDDM patients' sera to proteins in extracts of islets exposed to heat shock has failed to provide evidence for antibodies to islet heat shock proteins in the human disease (17). The aim of this study was to clarify whether the 50,000  $M_r$ , 40,000  $M_r$ , and 37,000  $M_r$  fragments of islet antigen are derived from distinct proteins and to investigate the relationships of these fragments to GAD and heat shock proteins.

## Methods

**Sera and antibodies.** Sera from recent onset IDDM patients, obtained within 10 d of diagnosis of disease, or from normal healthy controls, were selected from previous studies on antibodies to islet cell antigens (10, 13, 18). Sera were previously characterized for presence of antibodies to islet 64,000  $M_r$  proteins (18), to rat brain GAD (10), and to tryptic fragments of islet antigen (13). Specific antibodies to GAD and heat shock proteins were also used in antigen characterization. These

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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 antibody (1266) to a COOH-terminal 19-amino acid peptide of GAD<sub>67</sub> ([9], gift of Dr. J. Petersen, Hagedorn Research Laboratory, Gentofte, Denmark), a rabbit antibody (5551) to a 14-amino acid peptide (VTWNPHKMMGVPLQ) spanning the pyridoxal phosphate binding site of GAD<sub>65</sub> (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.

**Islet and insulinoma cell preparations.** Islets from 5–7-d-old neonatal Wistar rats were prepared as previously described (21). SV40-transformed hamster insulinoma cells (HIT-T15, gift of Dr. S. J. H. Ashcroft, University of Oxford, UK) and radiation-induced rat insulinoma cells (subclone RIN-5AH, 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 [<sup>35</sup>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 [<sup>35</sup>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<sub>3</sub>, 2 mg/ml bovine serum albumin (labeling medium) before labeling with [<sup>35</sup>S]methionine as described (3).

**Expression of recombinant GAD.** COS-7 fibroblast cells were transfected with cDNA for rat islet GAD<sub>67</sub> and endogenous proteins labeled with [<sup>35</sup>S]methionine as previously described (9). BHK cells were transfected with cDNA for human GAD<sub>65</sub> and GAD<sub>67</sub> (22) and maintained as stable cell lines, 64/11 and K87-8, respectively.

**Cellular extraction and immunoprecipitation.** Radiolabeled islets (2000–4000), insulinoma cells ( $2.5 \times 10^7$  cells) or COS cell transfectants were homogenized on ice by 20 passes of a motor-driven Teflon/glass homogenizer (Wheaton Instruments, Millville, NJ) in 600  $\mu$ l of 0.25 M sucrose, 10 mM Hepes (pH 7.4), 10 mM benzamidine, 0.1 mM *p*-chloromercuribenzenesulphonic 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 precleared with 50  $\mu$ l of normal human serum for 2 h at 4°C followed by binding to 100  $\mu$ l of protein A Sepharose (Sigma Immunochemicals) for 45 min at 4°C. A second preclearance was performed with 50  $\mu$ l of normal human serum for 18 h at 4°C and 100  $\mu$ l of protein A Sepharose for 45 min. Aliquots of extract representing  $5 \times 10^6$  cpm of radiolabeled protein in 50  $\mu$ l were incubated with 12.5  $\mu$ l of serum from type 1 diabetic patients or control individuals, or 2  $\mu$ l 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 isolated on 25  $\mu$ l (human sera) or 10  $\mu$ l (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  $\mu$ l 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*-chloro mercuribenzenesulphonic 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 benzamidine, 5 mM phenylmethylsulfonyl fluoride and twice in 3 ml of 10 mM Hepes (pH 7.4), 150 mM NaCl. Pellets were extracted in 12 ml of 10 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mg/ml trypsin for 30 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- $\mu$ l aliquots) were incubated with 12.5  $\mu$ l 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 antisera 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.

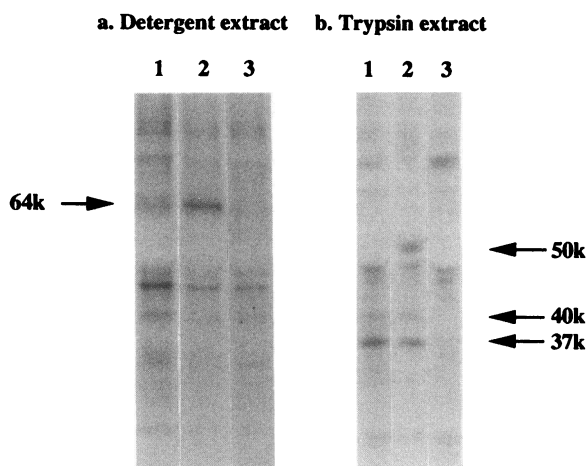
**Two-dimensional gel electrophoresis.** Islet extracts were processed for two-dimensional gel electrophoresis, with isoelectric focusing in the first dimension and SDS-PAGE in the second dimension, by the method of Hochstrasser et al. (24).

**Protein content and protein synthesis.** Protein contents of tissue homogenates were determined by the Biuret reaction (25). [<sup>35</sup>S]methionine incorporation into proteins was determined by spotting 5- $\mu$ l 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 *M<sub>r</sub>* 64,000 (Fig. 1 *a*, lane 1). These proteins were resolved as a diffuse doublet on SDS-PAGE and appeared distinct from the dominant 64,000 *M<sub>r</sub>* protein immunoprecipitated by sera containing GAD antibodies (Fig. 1 *a*, lane 2). Analysis of antibody binding to tryptic fragments of islet antigen indicated that these GAD-antibody-negative patients lacked antibodies to 50,000 *M<sub>r</sub>* fragments but possessed antibodies that recognized 37,000 and 40,000 *M<sub>r</sub>* fragments (Fig. 1 *b*, lane 1). Sera from patients positive for GAD antibodies immunoprecipitated 50,000 *M<sub>r</sub>* fragments from trypsin extracts of islets (Fig. 1 *b*, lane 2). These observations led us to investigate whether IDDM is associated with antibodies to 64,000 *M<sub>r</sub>* 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. 2 *a* 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 *M<sub>r</sub>* fragment of

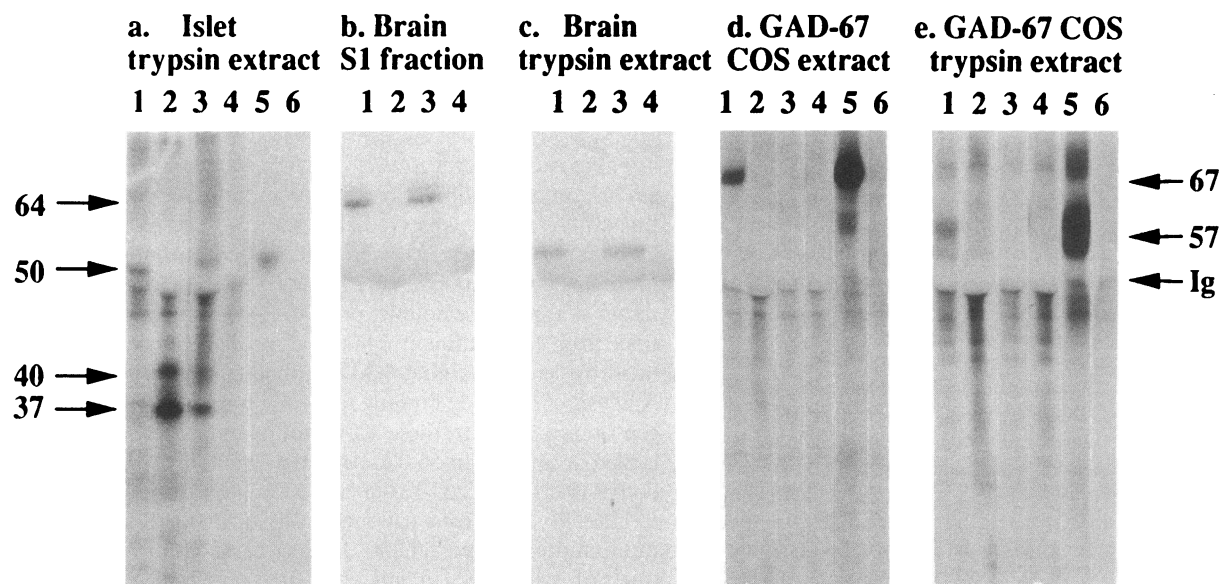


**Figure 1.** Autoantibody recognition of islet antigens. Sera from a GAD-antibody-negative IDDM patient (lanes 1), a GAD-antibody-positive patient (lanes 2) and a control subject (lanes 3) were used to immunoprecipitate proteins from detergent extracts (a) or trypsin extracts (b) of rat islets. Polypeptides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

antigen (Fig. 2 a, lane 1), antibodies to 37,000 and 40,000  $M_r$  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  $M_r$  fragments and failed to recognize either 40,000 or 37,000  $M_r$  fragments from the islet trypsin extract (Fig. 2 a, lane 5).

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 GAD<sub>67</sub>, which cross-reacts with GAD<sub>65</sub> (9). Serum from diabetic patients positive for antibodies to islet 50,000  $M_r$  tryptic fragments also immunoprecipitated 64,000  $M_r$  proteins with GAD immunoreactivity from soluble brain extracts (Fig. 2 b, lanes 1 and 3), and 50,000  $M_r$  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 GAD<sub>65</sub> (data not shown). Thus, trypsin extracts of brain contain 50,000  $M_r$  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  $M_r$  fragments recognized by these GAD antibodies.

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



**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 [<sup>35</sup>S]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<sub>67</sub> (d), and trypsin extracts of COS cells transfected with GAD<sub>67</sub> (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  $M_r$ s ( $\times 10^{-3}$ ) 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.

analysis of antibodies in 16 recent onset IDDM patients, 9 (62.5%) had antibodies that immunoprecipitated recombinant GAD<sub>67</sub>. No significant correlation was observed between the ability to immunoprecipitate GAD<sub>67</sub> from COS cell transfectants and GAD enzyme activity from rat brain extracts ( $r = 0.477$ ,  $P > 0.05$ ; Spearman correlation) or 37,000  $M_r$  tryptic fragments from islet extracts ( $r = 0.013$ ,  $P > 0.5$ ). Diabetic patients positive for antibodies to GAD<sub>67</sub>, and the sheep anti-serum to GAD, also immunoprecipitated polypeptides of  $\sim 57,000 M_r$  from trypsin extracts of the COS cell transfectants (Fig. 2 *e*, lanes 1 and 5).

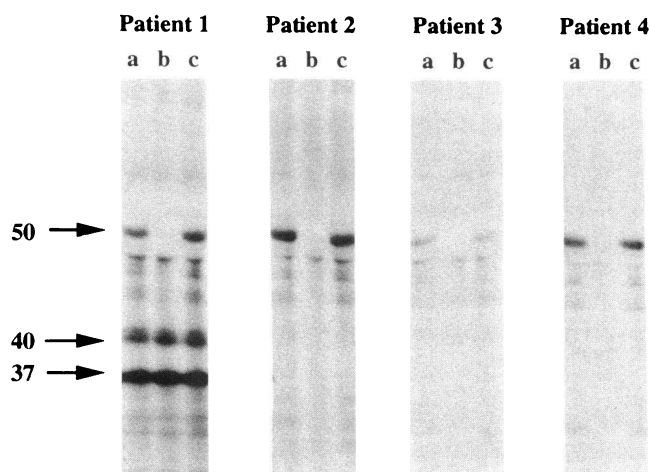
Studies were performed to determine whether recombinant human GAD<sub>65</sub> or GAD<sub>67</sub> can compete for binding with autoantibodies to rat islet antigens. Triton X-114 extracts of BHK cells containing the recombinant proteins were added to detergent phase extracts of [<sup>35</sup>S]methionine-labeled 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<sub>65</sub>, but not GAD<sub>67</sub>, blocked binding of antibodies to proteins generating 50,000  $M_r$  fragments on trypsinization (Fig. 3). GAD<sub>67</sub> was ineffective even with sera strongly positive for antibodies to recombinant GAD<sub>67</sub> (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  $2.28 \pm 0.28$   $\mu\text{mol}/16 \text{ h per g protein}$  ( $n = 3$ ) for HIT-T15 cells and

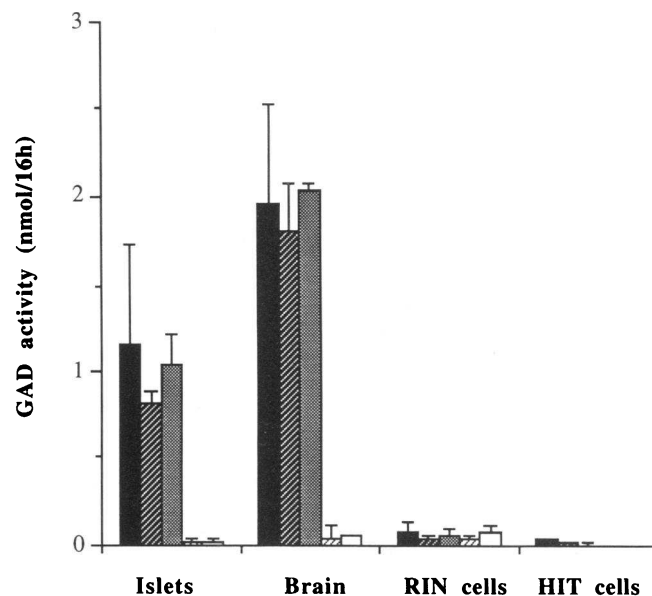
$3.04 \pm 0.53$   $\mu\text{mol}/16 \text{ h per g protein}$  ( $n = 3$ ) for RIN-5AH cells. In parallel experiments GAD enzyme activities in rat brain and neonatal rat islets were 104.26 and 9.52  $\mu\text{mol}/16 \text{ h per g protein}$ , respectively (10). Sera from three diabetic patients determined 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.

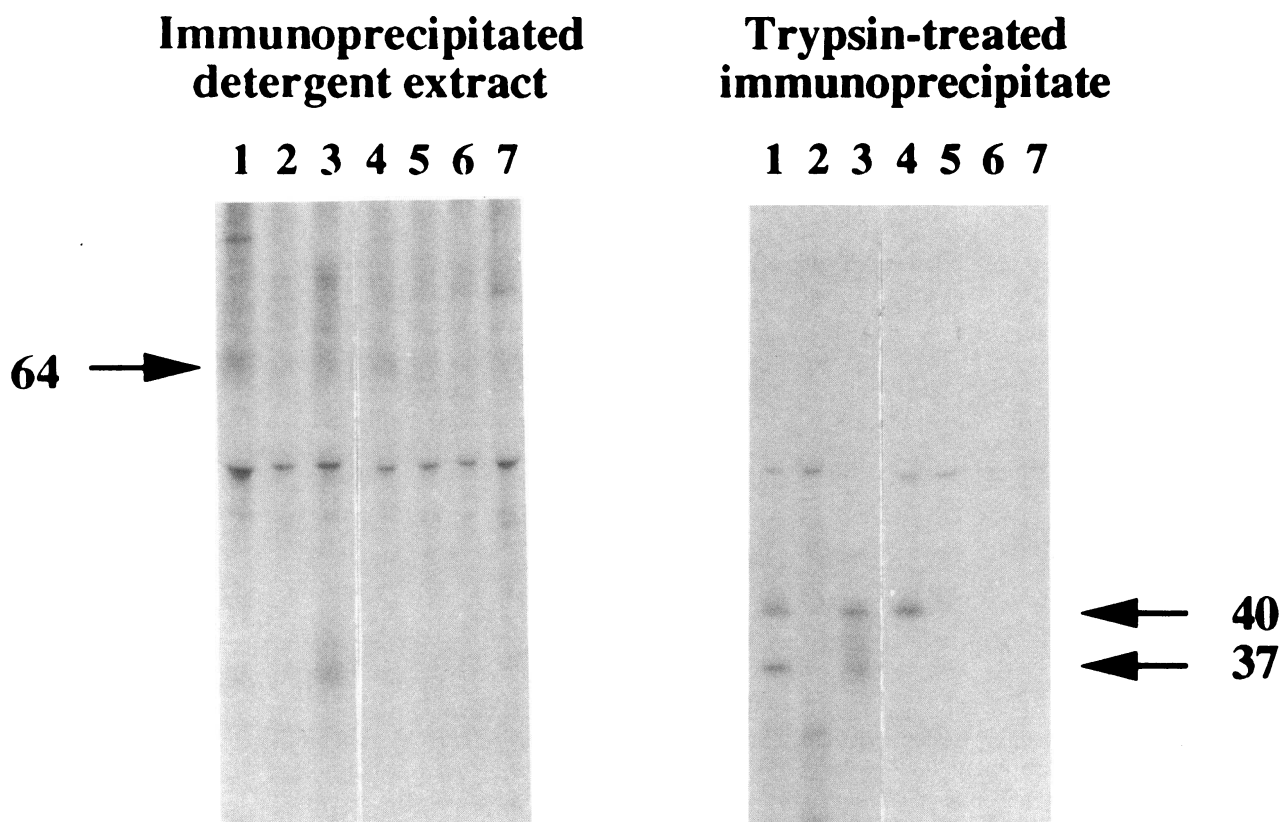
Competition experiments were performed to determine



**Figure 3.** Blocking of antibody binding to islet antigens with recombinant GAD. Triton X-114 detergent phase extracts of [<sup>35</sup>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  $3.3 \times 10^5$  cells) (b) BHK cell line (64/11) transfected with cDNA for GAD<sub>65</sub> ( $3.3 \times 10^4$  cells containing 7.2 nmol/2 h GAD), or (c) BHK cell line (87-11) transfected with cDNA for GAD<sub>67</sub> ( $2 \times 10^5$  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<sub>67</sub> (patient 1 in Fig. 2).



**Figure 4.** Immunoprecipitation of GAD activity from islets, brain, and insulinoma cell extracts. Nonidet P40 detergent-solubilized extracts (50  $\mu\text{l}$  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  $1.06 \pm 0.22$  (islets),  $2.19 \pm 0.37$  (brain),  $0.68 \pm 0.25$  (RIN cells) and  $3.58 \pm 0.40$  (HIT cells) nmol/16 h per 50  $\mu\text{l}$  ( $n = 3$ ). GAD activity in immunoprecipitates for each serum is shown. The results are mean  $\pm$ SEM of 3 independent observations.



**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  $M_r$ s ( $\times 10^{-3}$ ) of polypeptides immunoprecipitated by antibodies in IDDM patient's serum are marked.

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  $M_r$  fragments from [ $^{35}$ S]-methionine-labeled islet extracts but did not affect binding to 50,000  $M_r$  fragments. The insulinoma cells, therefore, contain proteins with similar antigenic properties to 37,000  $M_r$  and 40,000  $M_r$  islet fragments, but may lack proteins from which 50,000  $M_r$  fragments are derived.

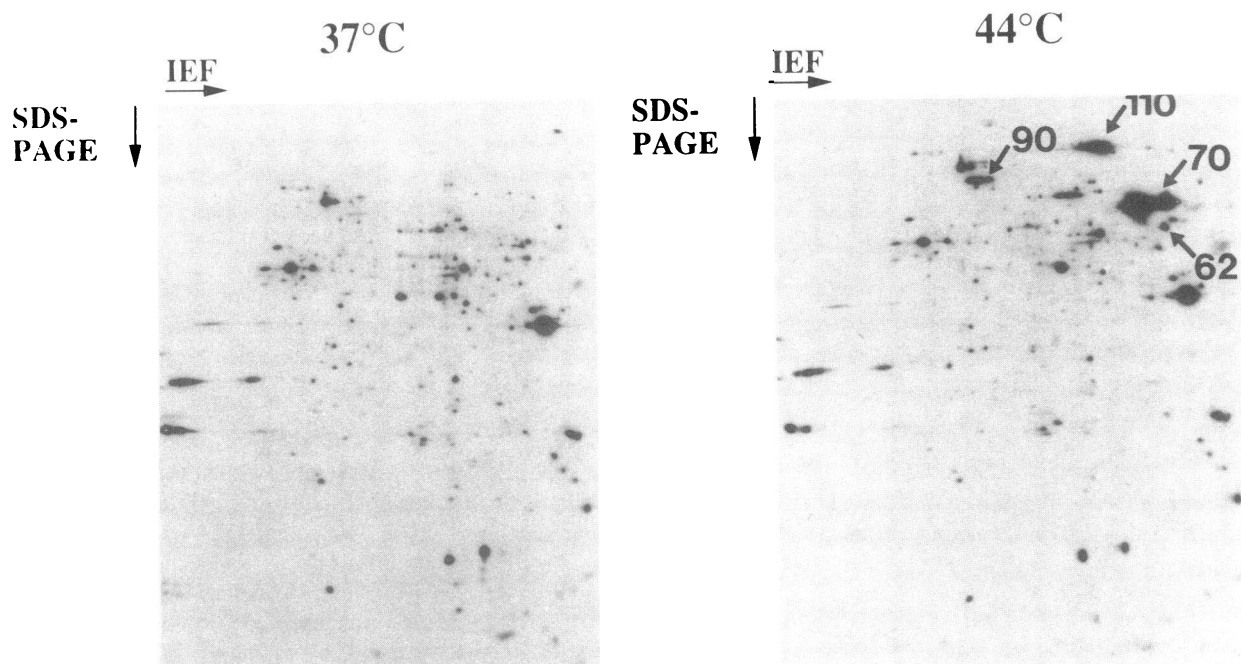
**Binding of antibodies to islet proteins after heat shock treatment.** To investigate possible relationships of islet 64,000  $M_r$  autoantigens to heat shock proteins, antibody reactivity to intact and trypsin-treated islet antigens was investigated in islets exposed to heat shock. Islets were incubated at 37°C (control) or 44°C (heat shock) for 20 min before labeling islet proteins with [ $^{35}$ S]methionine. Incorporation of [ $^{35}$ S]methionine into trichloroacetic acid-precipitable protein in heat shock treated islets was  $93.7 \pm 2.96\%$  (mean  $\pm$  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  $M_r$  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 centrifuga-

tion 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  $M_r$  and 70,000  $M_r$  islet proteins stimulated by heat shock treatment (Fig. 7). The 62,000  $M_r$  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  $M_r$  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  $M_r$  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  $M_r$  antigen migrated at a slightly higher  $M_r$  than the 62,000  $M_r$  hsp (Fig. 7). The recovery of radiolabeled 64,000  $M_r$  antigen was reduced in experiments with detergent phase extracts of islets preincubated at 44°C.

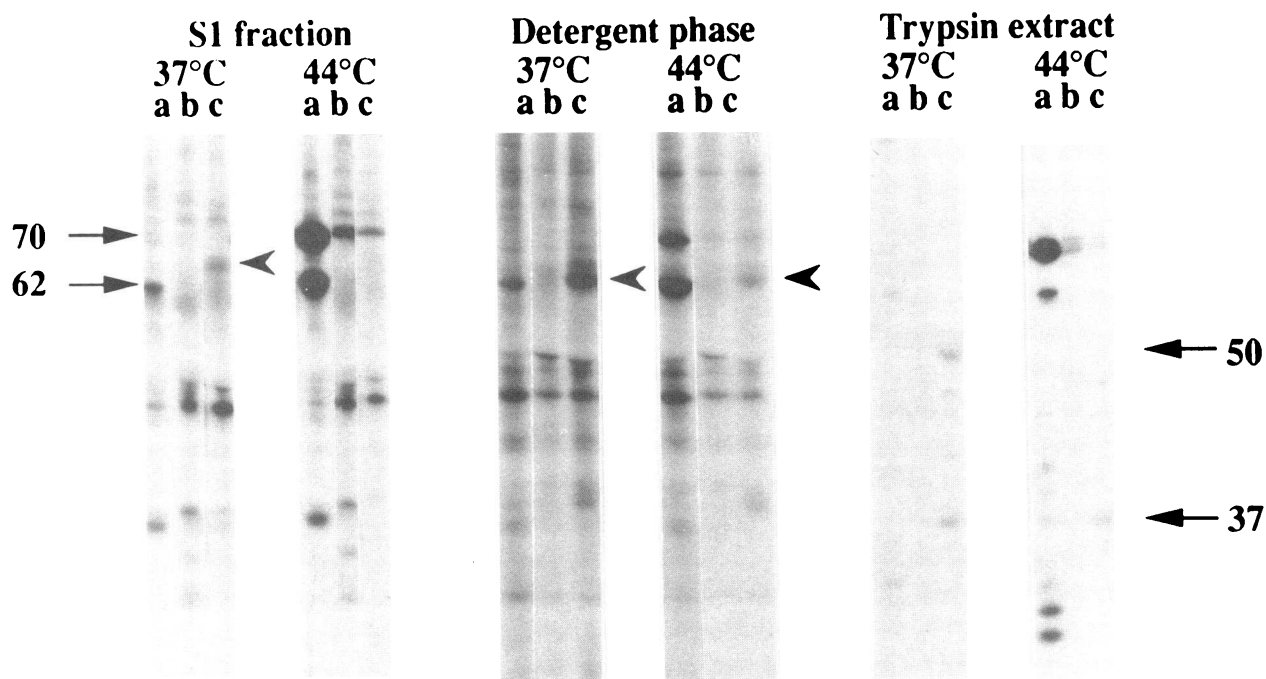
Trypsin solubilized extracts of islet particulate proteins were used to compare expression of precursors of antigenic



**Figure 6.** Islet protein expression after heat shock. Islets preincubated for 20 min at 37°C or 44°C were labeled with [<sup>35</sup>S]methionine for 5 h. Proteins in cytosolic (S1) fractions were separated by isoelectric focusing (IEF, *first dimension*) and SDS-PAGE (*second dimension*) and radio-labeled proteins detected by autoradiography. The  $M_r$ s ( $\times 10^{-3}$ ) of major proteins where [<sup>35</sup>S]methionine incorporation was stimulated by heat shock is indicated.

tryptic fragments after heat shock. The recovery of immuno-precipitable 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 [<sup>35</sup>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 auto-radiography. The  $M_r$ 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  $9.59 \pm 1.50\%$  (mean  $\pm$  SEM) of that under control conditions, whereas the density of 37,000  $M_r$  bands was  $89.9 \pm 10.7\%$  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  $M_r$  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  $M_r$  fragments and distinct antibodies that recognize common determinants on the 40,000 and 37,000  $M_r$  fragments. We have speculated that these fragments are derived from more than one 64,000  $M_r$  protein (13). GAD has been identified as a major 64,000  $M_r$  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  $M_r$  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  $M_r$  tryptic fragments of islet antigen (10). In this study, a polyclonal sheep antiserum raised to brain GAD immunoprecipitated 50,000  $M_r$  tryptic fragments that comigrated on SDS-PAGE with 50,000  $M_r$  fragments recognized by antibodies in IDDM. Furthermore, sera containing antibodies to 50,000  $M_r$  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  $M_r$  immunoreactive fragments from both islet and brain GAD, and these 55,000  $M_r$  polypeptides may be equivalent to the 50,000  $M_r$  fragments observed in our study. These data suggest that the 50,000  $M_r$  component of antigen is a tryptic polypeptide of GAD that includes the COOH-terminal domain of the enzyme. Addition of recombinant GAD<sub>65</sub> blocked binding of antibodies to proteins, generating 50,000  $M_r$  fragments on trypsinization. Addition of recombinant GAD<sub>67</sub> did not affect binding, even with sera shown to be strongly positive for antibodies to GAD<sub>67</sub>. The results suggest that the 50,000  $M_r$  fragment is derived from the lower  $M_r$  isoform of GAD, GAD<sub>65</sub>.

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

Since GAD<sub>67</sub> has also been reported to bind antibodies in IDDM (8, 9), we investigated whether the 37,000 and 40,000  $M_r$  fragments are derived from this second GAD isoform. Our

findings suggest that GAD<sub>67</sub> is not a target for 37k antibodies. The 37,000 and 40,000  $M_r$  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 GAD<sub>65</sub> nor GAD<sub>67</sub> competed for autoantibody binding to precursors of 37,000 and 40,000  $M_r$  fragments. No correlation was observed between the ability of antibodies to recognize recombinant GAD<sub>67</sub> and the 37,000/40,000  $M_r$  tryptic fragments and trypsinization of recombinant GAD<sub>67</sub> generated 57,000  $M_r$  polypeptides and not 37,000 and 40,000  $M_r$  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 GAD<sub>65</sub> or GAD<sub>67</sub> in RIN and HIT cells, precursors to 37,000 and 40,000  $M_r$  antigenic fragments were clearly present in these cells. Together, these results provide strong evidence that the 37,000 and 40,000  $M_r$  fragments are derived from proteins distinct from both GAD<sub>65</sub> and GAD<sub>67</sub>.

The precursors of the 37,000 and 40,000  $M_r$  antigen fragments do appear to be proteins of  $M_r$  64,000. Diffuse bands at this  $M_r$  were immunoprecipitated from islet extracts by GAD-antibody-negative, 37k-antibody-positive sera. We, and others, have consistently failed to detect proteins of  $M_r$  other than 64,000 specifically immunoprecipitated by IDDM sera (28). Two-dimensional electrophoresis of the eluted immune complexes resolves 64,000  $M_r$  proteins as multiple spots; two components ( $\alpha$  and  $\beta$ ) display charge heterogeneity and have identical mobility on gels to  $\alpha$  and  $\beta$  components of GAD<sub>65</sub> (6, 28). A third protein, designated  $\gamma$ , with identical  $M_r$  to the  $\beta$  component, but more negatively charged, has also been reported (29). Analysis of the relationship of the  $\gamma$ -component to 37,000 and 40,000  $M_r$  fragments requires specific antibodies to the protein, which are currently not available.

Diffuse bands of  $M_r$  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 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  $M_r$  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  $M_r$  proteins immunoprecipitated by antibodies in diabetes migrated at a slightly higher  $M_r$  than a 62,000  $M_r$  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  $M_r$  fragments do not appear to be related to heat shock proteins, since [ $^{35}\text{S}$ ]-methionine incorporation into 37,000  $M_r$  antigenic fragments was not stimulated by heat shock. Polyclonal antibodies to hsp 65 did not cross-react with 37,000  $M_r$  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 GAD<sub>65</sub> 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  $M_r$  protein autoantigen in IDDM and antibodies to 37,000 and 40,000  $M_r$  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  $M_r$  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  $M_r$  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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