Replacement of methionine-161 with threonine eliminates a major by-product of human glutamate decarboxylase 65-kDa variant expression in *Escherichia coli*

Javier Santos*, Erica A. Antón*, Cristina Marino-Buslje*, Silvina N. Valdez†, Ana L. Villanueva†, Mauricio Sica†, Rubén Iacono†, Paulo Maffia*, Edgardo Poskus† and Mario R. Ermácora*[1]

**Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Argentina, Roque Sáenz Peña 180, (1876) Bernal, Argentina, and †Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, and IDEHU-CONICET, Buenos Aires, Argentina*

Most insulin-dependent diabetes mellitus patients generate conformational autoantibodies to the islet-cell 65-kDa variant of human glutamate decarboxylase (GAD65), and several immunochemical tests for the early detection of type-1 diabetes rely on GAD65 antibody (GADA) assessment using properly folded recombinant GAD65 as the antigen. In addition, preventive therapies based on tolerization by GAD65 administration may be available in the near future. Therefore, there exists a strong interest in a facile and economically sound expression procedure for this antigen. Several attempts to produce, in native form, wild-type GAD65 in *Escherichia coli* have failed. However, this difficulty was recently surmounted in our laboratory by expressing GAD65 as a fusion protein with thioredoxin [Papouchado, Valdez, Ghiringhelli, Poskus and Ermácora (1997) Eur. J. Biochem. 246, 350–359]. In this work, a new GAD65 hybrid gene was prepared by joining engineered cDNA obtained from human and rat tissues. The new gene was modified additionally to finally code for human GAD65 with a single amino-acid substitution: Met-161 → Thr. This change impeded the co-expression of a 48-kDa by-product from an internal translation site. Also, a second 58-kDa by-product was identified as a GAD65 C-terminal proteolytic fragment that co-purifies with thioredoxin–M161T GAD65. The new GAD65 variant was expressed and easily purified, yielding an antigen that performed equally or better than wild-type GAD65 in the reference radiobinding assay for GADA. The procedure provides an inexpensive source of large amounts of fully active and immunochemically competent GAD65.

Introduction

Autoimmunity is a vast health problem. Millions of patients suffer from metabolic and physiological disorders due to a malfunction of the immune system that results in attack to self-antigens. The identification, large-scale production and application of autoantigens are major goals of biotechnological research. Among these autoantigens, the 65-kDa variant of glutamate decarboxylase (GAD65; EC 4.1.1.15) stands prominently because it is recognized by T-cells and also by GAD65 autoantibodies (GADA) from patients suffering type-1 diabetes, polyendocrine autoimmune syndrome and stiff-man syndrome [1–5].

GADA can be found in most patients suffering type-1 diabetes (insulin-dependent diabetes) several years before the appearance of clinical symptoms. Therefore, an international effort is taking place to develop efficient and widely applicable GADA immunochemical tests that, in combination with other diagnostic tools, would help to detect individuals at risk and enroll them in prevention programs [6]. In addition, it has been found recently that GADA in adults with type-2 diabetes (the most prevalent type of diabetes) and with gestational diabetes are markers for progression to insulin dependence [7–10], offering the opportunity to improve the control and treatment of these patients.

GAD65 is a complex molecule with a prosthetic group (pyridoxal 5′-phosphate) and 15 cysteine residues. Its native conformation is essential for an appropriate GADA assay because most GADA are directed against native epitopes [11,12]. Native recombinant GAD65 can be obtained from eukaryotic expression systems [13–16]. However, the large-scale production of a recombinant protein in eukaryotic systems is more expensive and technically complex than in

Key words: autoantibodies, insulin-dependent diabetes mellitus, genetic engineering, protein expression.

Abbreviations used: GAD65, 65-kDa variant of glutamate decarboxylase; GADA, GAD65 autoantibodies; Trx, *Escherichia coli* thioredoxin; RBA, radiobinding assay; SEC, size-exclusion chromatography; As-matrix, phenylarsine oxide-containing matrix.

[1] To whom correspondence should be addressed.

The cDNA sequence coding for residues 167–585 of human GAD65 glutamate decarboxylase has been submitted to the EMBL, GenBank®, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AJ251501.

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Escherichia coli. Moreover, in eukaryotic cells, the enzyme appears in different cellular compartments and suffers post-translational modifications that are not needed for the enzymic and immunochemical activity [17], all of which complicates the production of pure GAD65. Unfortunately, previous attempts to express properly folded in *E. coli* were unsuccessful because extensive aggregation and misfolding precluded the isolation of soluble enzyme [18,19]. We recently found a solution to this problem by expressing GAD65 as a fusion protein with *E. coli* thioredoxin (Trx), Trx–GAD65 [19]. Also, to test GADA, we developed an efficient and highly specific ELISA that benefits from the Trx moiety of Trx–GAD65 [20]. Now, as part of an institutional program aimed at building and optimizing a collection of cDNAs coding for antigens that might have a strong impact in preventive medicine, our laboratory undertook the cloning of the *GAD65* gene and its genetic engineering to improve GAD65 production in *E. coli*. The first results of this effort are reported here.

Materials and methods

General details

Serum samples from patients with type-1 diabetes were obtained from the Research Institute for Children, Harahan, LA, U.S.A., for the 4th International GADA Proficiency Test Survey. The normal controls were healthy individuals with no personal or familial history of type-1 diabetes. SDS/PAGE, protein blotting to PVDF membranes, and sequencing were performed as described in [21–23]. A dye-binding method was used for protein-concentration measurements [24]. Unless otherwise indicated, standard molecular-biology protocols were used [25].

Antibodies, enzymes and expression vectors

The monoclonal antibody GAD6 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.) was purified from ascitic fluid by FPLC using a Mono Q-Sepharose column (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and a continuous gradient (from 0 to 1 M NaCl in 0.02 M Tris/HCl, pH 8.5).

Restriction enzymes, *Taq* DNA polymerase and T4 ligase were from Promega (Madison, WI, U.S.A.). A complete list of the oligonucleotides utilized as primers is given in Figure 1. The preparation of the expression vector pGAD65(Trx), which codes for wild-type human GAD65 [26,27], was described previously [19]. pTrxFus was provided by Invitrogen (San Diego, CA, U.S.A.). pSP64 poly(A) was provided by Promega.

Isolation and engineering of GAD65 cDNA

Total RNA was prepared by extraction with phenol and guanidinium thiocyanate [28]. Bioptic tissue of human pancreas was obtained at the Hospital General de Agudos Cosme Argerich, Buenos Aires, Argentina. A male Wistar rat killed by ether vapours was the source of brain tissue. Moloney murine leukaemia virus reverse transcriptase

Primer	Sequence
3'GAD	tctagattattataaatcttgtccaagg
5'GAD-nou	tctccgggctctggcttttggtctttcg
r880	gactatgttcagacgtgaaggca
r624	cagccagtctgctgctaatccaacc
XBAI-T15	gtcgactctagattttttttttttttt
5'M161T	ggaggaaattttgacccattgccaaacaac
3'GAD	tctagattattataaatcttgtccaagg
5'GADKpn	tttggtacccatggcatctccgggctctggcttttggtctt
3'GADAge	tccaaccatatccaaaccggtagacag
R1USr	cttccgggcggcggcccgcggcggctggctcccgccgctctctgctggcttctcagcgtctcc
R1DSf	ggccgccgcccggaaggccgcctgcgcctgtgaccaaaaccctgcagctcgtccaaagtagatgtca
R2USr	atcacaggctggcagcaggtctgttgcgtggagaaatgcataattgaca
R2DSf	tttctccacgcaacagacctgctgccagcctgtgatggagaaaggccca
AGEI(C)	tctaccggtttggatatggttggactagcagca
R3f	tttggtacccatggcatctccgggctctggcttttggtctttcggatctgaagatggctctggggattctgagaacccgggaaca
5' XbaI	tgacatctagaccatggcatctccgggctc
3'TOTAL	attactgcaggtcgactctagat

Figure 1 Primers utilized for reverse transcription and PCR mutagenesis

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(Promega) was used to synthesize cDNA [29]. The resulting single-stranded cDNA was used as template in double-stranded-cDNA synthesis by PCR [30]. PCR products were cloned into the pGEMT Easy Vector System or pGEM3Zf (Promega) following the manufacturer's instructions. PCR engineering of DNA was performed according to a general procedure described previously [31]. The *Pfu* DNA polymerase used was from Stratagene (La Jolla, CA, U.S.A.). DNA sequencing was performed at the Cancer Research Center, Chicago University (Chicago, IL, U.S.A.).

Expression of GAD65 variants

Wild-type GAD65 and M161T GAD65 (a GAD65 variant in which Met-161 had been replaced by Thr) were transcribed and translated in the presence of [^{35}S]methionine (New England Nuclear, Boston, MA, U.S.A.) using a rabbit reticulocyte lysate system (Promega) as described in [19,20].

The expression in *E. coli* and subsequent purification of Trx–GAD65 and Trx–M161T GAD65 were performed essentially as described in [19]. Briefly, *E. coli* GI 698 carrying the corresponding expression vectors was cultured at 30 °C in M9 salts (0.2% casamino acids, 0.5% glucose, 1 mM $MgCl_2$ and 100 μg/ml ampicillin). Protein expression was induced overnight with 100 μg/ml tryptophan at 20 °C. For expression at 37 °C, *E. coli* GI 724 was used instead. After the induction, bacteria from 200 ml of culture were collected by centrifugation, resuspended in lysis buffer (4 ml; 50 mM Tris/HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1% Aprotinin and 2 mM PMSF) and sonicated. After this, Triton X-100 was added to a final concentration of 0.1% and the incubation was continued for 10 min at 0 °C. The soluble intracellular fraction was isolated by centrifugation (12000 ***g***, 15 min, 4 °C). The Trx-binding matrix ThioBond (2 ml; Invitrogen), equilibrated with lysis buffer, was added to the soluble fraction (4 ml) and the suspension was incubated for 1 h at 4 °C. The suspension was poured into a column (0.7 cm × 9.0 cm) and washed first with 6 vol. of 1 mM 2-mercaptoethanol and then with 3 vols of 5 mM 2-mercaptoethanol. Bound Trx–GAD65 was eluted with 4 ml of lysis buffer containing 100 mM 2-mercaptoethanol. GAD65 was further purified by size-exclusion chromatography (SEC) using an FPLC system equipped with a Superose 12 column (Pharmacia-LKB Biotechnology) calibrated with a gel-filtration standard kit from Bio-Rad (Hercules, CA, U.S.A.). The elution solvent was 50 mM sodium phosphate/100 mM NaCl, pH 7.0.

GADA measurements

GADA titre (GAD Index) in serum samples was determined using the reference radiobinding assay (RBA). This procedure was fully described previously [19,20,32] and validated in the second and third (Immunology Diabetes Workshop, University of Florida, FL, U.S.A., 1996 and 1997), and in the fourth (Research Institute for Children, Harahan, LA, U.S.A., 1998) GADA Proficiency Tests, in which our laboratory achieved the highest possible score in all parameters (sensitivity, validity, specificity and consistency). Only a brief outline of the procedure is given here. [^{35}S]GAD65 reticulocyte lysate translation products were diluted in RBA buffer (0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4, 0.15% Tween 20, 0.1% Aprotinin and 0.1% BSA) and applied to a PD10 column (Pharmacia–LKB Biotechnology) to remove free [^{35}S]methionine. [^{35}S]GAD65 variants were incubated overnight at 4 °C with the serum samples. Subsequently, Protein A–Sepharose was added and the immunocomplexes formed were collected by centrifugation. The pellets were washed, dissolved in 1% SDS, and transferred to vials for scintillation counting. GAD Index was calculated as (c.p.m.$_{\text{unknown}}$−c.p.m.$_{\text{control}}$)/(c.p.m.$_{\text{standard}}$−c.p.m.$_{\text{control}}$), where c.p.m.$_{\text{control}}$ is the average c.p.m. reading for the healthy controls included in the assay and c.p.m.$_{\text{standard}}$ is the reading of an international GADA-positive standard [32].

Results and discussion

Genetic engineering of the *GAD65* gene

Several attempts to isolate the entire human *GAD65* gene from pancreatic tissues by reverse transcriptase PCR were unsuccessful; however, the 3′ end of the gene (bp 468–1758, Figure 2) was amplified with primers 5′M161T and 3′GAD (Figure 1). The resulting DNA sequence matched previously published data [26,33,34] except for bases 571 and 1014 (Figure 2). These replacements did not alter the translated amino acid sequence. Since the original pancreatic tissue was used entirely in the first cDNA preparation, it was impossible to establish if the observed changes corresponded to an evolutionary variant of human GAD65 or were the product of base mis-incorporation by the polymerase. To facilitate further mutagenesis (see below), an *AgeI* site was introduced at position 550 by replacing thymidine 552 with cytidine, which did not alter the amino acid sequence of the translation product.

To obtain the 5′ end of the gene (bp 7–588, Figure 2), rat brain RNA was retrotranscribed using the primer 3′GAD. The resulting product was amplified by PCR using primers 5′GAD-nou and r880 (see Figure 1), and used as template in a second amplification with primers 5′GAD-nou and r624. The sequence of the product matched data published previously for the 5′ end of the rat *GAD65* gene (Figure 2) [35]. The fragment produced differed from the human sequence at 69 bp, which were scattered along the entire segment. To humanize the rat cDNA fragment, three consecutive rounds of PCR mutagenesis were per-

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              10         20         30         40         50         60         70         80         90        100
r      ATGGCATCTC CGGGCTCTGG CTTTTGGTCc TTCGGATCTG AAGATGGCTC TGGGGATcCT GAGAACCCGG GAACAGCGAG AGCCTGGTGC CAGGTGGCCC
rh     ATGGCATCTC CGGGCTCTGG CTTTTGGTCT TTCGGaTCtG AAGATGGCTC TGGGGATTCt GAGAAcCCgG GaACAGCGaG AGCCTGGTGC CAgGTGGCcC
h      ATGGCATCTC CGGGCTCTGG CTTTTGGTCT TTCGGGTCGG AAGATGGCTC TGGGGATTCC GAGAATCCCG GCACAGCGCG AGCCTGGTGC CAAGTGGCTC
        M  A  S   P  G  S  G   F  W  S   F  G  S   E  D  G  S   G  D  S   E  N  P   G  T  A  R   A  W  C   Q  V  A

r    1 AAAAGTTCAC GGGCGGCATC GGAAACAAGC TATGCGCTCT GCTCTACGGA GACtCTGAGA AGCCAGCAGA GAGCGGCGGG AGCgtGaCct CGCGGGCCGC
rh     AaAAGTTCAC GGGCGGCATC GGAAACAAgC TaTGCGCtCT GCTCTACGGA GACGCtGAGA AGCCaGCaGA GAGCGGCGGG AGCCAgCCgC CGCGGGCCGC
h      AGAAGTTCAC GGGCGGCATC GGAAACAAAC TGTGCGCCCT GCTCTACGGA GACGCCGAGA AGCCGGCGGA GAGCGGCGGG AGCCAACCCC CGCGGGCCGC
       Q  K  F  T   G  G  I   G  N  K   L  C  A  L   L  Y  G   D  A  E   K  P  A  E   S  G  G   S  Q  P   P  R  A  A

r    2 CaCtCGGAAG GtCGCCTGCa CCTGTGACCA AAAACCCTGC AGCTGCcCCA AAGgAGATGT CAATTATGCA cTTCTCCACG CAACAGACCT GCTGCCAGCC
rh     CGCCCGGAAG GCCGCCTGCG CCTGtGACCA aAAaCCCTGC AGCTGCTCCA AAGTaGATGT CAAtTAtGCa TTTCTCCAcG CAACAGACCT GCTGCCaGCc
h      CGCCCGGAAG GCCGCCTGCG CCTGCGACCA GAAGCCCTGC AGCTGCTCCA AAGTGGATGT CAACTACGCG TTTCTCCATG CAACAGACCT GCTGCCGGCG
         A  R  K   A  A  C   A  C  D  Q   K  P  C   S  C  S   K  V  D  V   N  Y  A   F  L  H   A  T  D  L   L  P  A

r    3 TGTGAaGGAG AAAGGCCCAC TCTCGCATTT CTGCAAGATG TAATGAACAT TTTGCTTCAG TACGTGGTGA AAAGTTTTGA TAGATCAACT AAaGTGATTG
rh     TGTGATGGAG AAAGGCCCAC TcTcGCaTTT CTGCAAGATG TaATGAACAT TTTgCTTCAG TAcGTGGTGA AAAGTTTtGA TAGATCAACt AAgGTGATTG
h      TGTGATGGAG AAAGGCCCAC TTTGGCGTTT CTGCAAGATG TTATGAACAT TTTACTTCAG TATGTGGTGA AAAGTTTCGA TAGATCAACC AAAGTGATTG
        C  D  G   E  R  P  T   L  A  F   L  Q  D   V  M  N  I   L  L  Q   Y  V  V   K  S  F  D   R  S  T   K  V  I

r    4 ATTTCCATTA CCCCAATGAG CTTCTTCAAG AGTATAATTG GGAATTGGCA GACCAACCGC AAAATCTGGA GGAAATTTTG ACGCACTGCC AAACAACTCT
rh     ATTTCCATTA cCCcAATGAG CTTCTtCAAG AgTATAATTG GGAATTGGCA GACCAACCgC AAAATcTGGA GGAAATTTTG AcGCAcTGCC AAACAACTCT
h      ATTTCCATTA TCCTAATGAG CTTCTCCAAG AATATAATTG GGAATTGGCA GACCAACCAC AAAATTTGGA GGAAATTTTG ATGCATTGCC AAACAACTCT
       D  F  H  Y   P  N  E   L  L  Q   E  Y  N  W   E  L  A   D  Q  P   Q  N  L  E   E  I  L   T  H  C   Q  T  T  L

r    5 AAAATATGCG ATTAAAACAG GGCATCCCCG ATATTTTAAT CAGCTGTCTA CCGGaTTGGA TATGGTTGGA tTAGCAGCAG AtTGGtTGAC ATCAACAGCA
rh     AAAATATGCg ATTAAAACAG GGCATCCccG ATAtTTtAAT CAgCTgTCTA CcGGTTTGGA TATGGTTGGA cTAGCAGCAG ACTGGCTGAC ATCAACAGCA
h      AAAATATGCA ATTAAAACAG GGCATCCTAG ATACTTCAAT CAACTTTCTA CTGGTTTGGA TATGGTTGGA TTAGCAGCAG ACTGGCTGAC ATCAACAGCA
         K  Y  A   I  K  T   G  H  P  R   Y  F  N   Q  L  S   T  G  L  D   M  V  G   L  A  A   D  W  L  T   S  T  A

r    6 AAcACgAACA TGTTtACCTA TGAgATcGCc CCtGTATTTG TaCTacTGGA ATATGTgACA CTAAAGAAAA TGAGgGAAAT CATTGGCTGG CCAGGaGGCT
rh     AATACTAACA TGTTCACCTA TGAAATTGCT CCAGTATTTG TGCTTTTGGA ATATGTCACA CTAAAGAAAA TGAGAGAAAT CATTGGCTGG CCAGGGGGCT
h      AATACTAACA TGTTCACCTA TGAAATTGCT CCAGTATTTG TGCTTTTGGA ATATGTCACA CTAAAGAAAA TGAGAGAAAT CATTGGCTGG CCAGGGGGCT
        N  T  N   M  F  T  Y   E  I  A   P  V  F   V  L  L  E   Y  V  T   L  K  K   M  R  E  I   I  G  W   P  G  G

              10         20         30         40         50         60         70         80         90        100
rh   7 CTGGCGATGG GATATTTTCT CCCGGTGGCG CCATATCTAA CATGTATGCC ATGATGATCG CACGCTTTAA GATGTTCCCA GAAGTCAAGG AGAAAGGAAT
     8 GGCTGCTCTT CCCAGGCTCA TTGCCTTCAC GTCTGAACAT AGTCATTTTT CTCTCAAGAA GGGAGCTGCA GCCTTAGGGA TTGGAACAGA CAGCGTGATT
     9 CTGATTAAAT GTGATGAGAG AGGGAAAATG ATTCCATCTG ATCTTGAAAG AAGGATTCTT GAAGCCAAAC AGAAAGGGTT TGTTCCTTTC CTCGTGAGTG
    10 CCACAGCTGG AACtACCGTG TACGGAGCAT TTGACCCCCT CTTAGCTGTC GCTGACATTT GCAAAAAGTA TAAGATCTGG ATGCATGTGG ATGCAGCTTG
    11 GGGTGGGGGA TTACTGATGT CCCGAAAACA CAAGTGGAAA CTGAGTGGCG TGGAGAGGGC CAACTCTGTG ACGTGGAATC CACACAAGAT GATGGGAGTC
    12 CCTTTGCAGT GCTCTGCTCT CCTGGTTAGA GAAGAGGGAT TGATGCAGAA TTGCAACCAA ATGCATGCCT CCTACCTCTT TCAGCAAGAT AAACATTATG
    13 ACCTGTCCTA TGACACTGGA GACAAGGCCT TACAGTGCGG ACGCCACGTT GATGTTTTTA AACTATGGCT GATGTGGAGG GCAAAGGGGA CTACCGGGTT
    14 TGAAGCGCAT GTTGATAAAT GTTTGGAGTT GGCAGAGTAT TTATACAACA TCATAAAAAA CCGAGAAGGA TATGAGATGG TGTTTGATGG GAAGCCTCAG
    15 CACACAAATG TCTGCTTCTG GTACATTCCT CCAAGCTTGC GTACTCTGGA AGACAATGAA GAGAGAATGA GTCGCCTCTC GAAGGTGGCT CCAGTGATTA
    16 AAGCCAGAAT GATGGAGTAT GGAACCACAA TGGTCAGCTA CCAACCCTTG GGAGACAAGG TCAATTTCTT CCGCATGGTC ATCTCAAACC CAGCGGCAAC
    17 TCACCAAGAC ATTGACTTCC TGATTGAAGA AATAGAACGC CTTGGACAAG ATTTATAA
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Figure 2 Sequences of rat (r), human (h) and hybrid (rh) *GAD65* genes

The changes engineered into rat *GAD65* to produce rat–human GAD65 and the subsisting differences between the hybrid and human constructions are shown in bold lower case. The rat–human GAD65 construct codes for the wild-type human amino acid sequence except for Met-161, which was replaced by Thr to disrupt an internal translation site. Some of the bases were modified to introduce restriction sites. The 3′ end of the rat–human *GAD65* gene (bp 468–1758) was retrotranscribed from human RNA and its sequence was in agreement with previous reports [26,33] with the exception of changes at positions 571 and 1014, which did not modify the codon specificity. The first 230 amino acids of human GAD65 are shown.

formed, as described schematically in Figure 3. The result was a rat–human hybrid nucleotide sequence (1–555, Figure 2) that coded for the first 185 amino acids of human GAD65 except for a Met-161 → Thr replacement. Thr is found at position 161 in the rat and mouse GAD65 and is a conservative replacement. We decided to leave Thr-161 unchanged to disrupt an internal initiation site of translation that was shown in our previous work to produce a 48-kDa N-terminally truncated GAD65 [19].

The above-described 5′ and 3′ fragments of the *GAD65* gene were cloned consecutively and in-frame into pTrxFus to yield pRH1GAD65(Trx) (Figure 4). The new expression vector differed from our previous construction, pGAD65(Trx) [19], at 64 bp positions (55 bp within the *GAD65* coding sequence and in 9 bp in the spacer linking Trx to GAD65). However, in terms of amino acid sequence, the Trx–GAD65 fusion protein coded for by pRH1GAD65(Trx) only contained a single change in the GAD65 moiety (Thr instead of Met at position 161) and an extra tripeptide (VPM, Figure 4) in the linker to Trx.

Expression of GAD65 from pRH1GAD65(Trx) in *E. coli*

E. coli cells transformed with pRH1GAD65(Trx) were used to produce M161T GAD65 at 30 and 37 °C. As judged by comparative SDS/PAGE of *E. coli* extracts, this variant was produced at high yields (≈ 10% of the total proteins). Although a large fraction of the expressed GAD65 was sequestered in inclusion bodies, ≈ 5 mg of active enzyme per litre of culture was recovered soluble (≈ 1% of the total *E. coli* protein). These results are essentially identical to those reported before for Trx–GAD65 [19].

In the first purification step, the Trx fusion protein present in the soluble cellular fraction was bound to a phenylarsine oxide-containing matrix (As-matrix) specific for the Trx active site. Proteins bound non-specifically were eliminated by washing with 1 mM 2-mercaptoethanol (first wash) and then with 5 mM 2-mercaptoethanol (second wash). Bound Trx–M161T GAD65 was eluted with 0.1 M 2-mercaptoethanol. The second purification step was a SEC. The profile of this separation is shown in Figure 5. The

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Figure 3 Outline of the general procedure employed to construct the 5′ end of the gene coding for M161T GAD65

A cDNA fragment spanning bp 1–555 of the rat *GAD65* gene (Figure 2) inserted into pGEM3Zf was the starting material. Three consecutive rounds of PCR mutagenesis [31] were performed. A list of the primers utilized is presented in Figure 1. As an example, the first mutagenesis round is shown. The second round was identical except that primers R1US_r and R1DS_f were replaced by R2US_r and R2DS_f, respectively. The third round of mutagenesis consisted of a single PCR with primers R3_f and 3′GADAge. After each round, the PCR product was cloned into the *Sma*I site of pGEM3Zf for preparative production and sequencing. The part of the sequence changed in each round is shown within a box. All the changes are summarized in Figure 2.

Figure 5 SEC of affinity-purified Trx–M161T GAD65

The separation was performed on a calibrated Superose 12 column. Peak A, high-molecular-mass aggregates; peak B, dimeric Trx–M161T GAD65; peak C, 2-mercaptoethanol and low-molecular-mass compounds.

purification was evaluated by measuring total protein and specific activity, and by SDS/PAGE and Western blotting with GAD6, a monoclonal antibody directed against a linear C-terminal epitope of GAD65. The results were in excellent agreement with our previous results with Trx–GAD65 [19] and indicated that the two variants equally purified to a > 90% pure Trx–GAD65 preparation, yielding the expected 77-kDa protein and a 58-kDa GAD65 fragment in approximately a 3:2 ratio (see below).

Comparison of GAD65 and M161T GAD65

The specific enzymic activity of Trx–M161T GAD65, determined by measuring the release of [^{14}C]CO_2 from [^{14}C]glutamic acid, was 3.2 units/mg. Within experimental error, this value was identical to the specific activity of Trx–GAD65 [19], which indicates that the Met-161 → Thr substitution and the changes in the Trx–GAD65 linker did not alter the conformational integrity of the molecule significantly.

Figure 4 Simplified restriction map of the Trx–M161T GAD65 fusion protein showing the nucleotide and amino acid sequences at the junction between Trx and M161T GAD65

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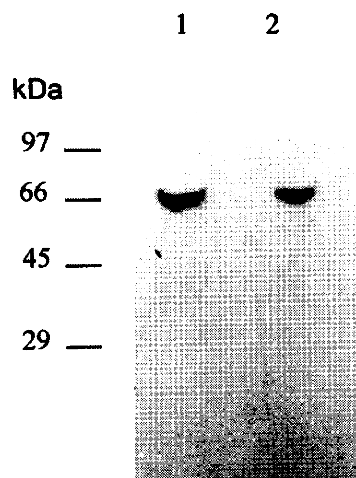

Figure 6 SDS/PAGE and fluorography of the ^{35}S-labelled expression products in a rabbit reticulocyte lysate system of M161T GAD65 (lane 1) and wild-type GAD65 (lane 2)

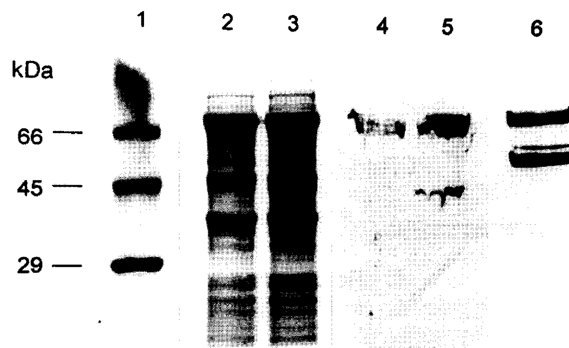

Figure 8 SDS/PAGE analysis of GAD65 expression products in *E. coli*

Total lysates of cells expressing M161T GAD65 (lanes 2) and wild-type GAD65 (lane 3) stained with Coomassie Brilliant Blue. Western blotting of total cell lysates and immunostaining with GAD6 (an antibody that recognizes a linear epitope in the C-terminus of GAD65): M161T GAD65 (lane 4) and wild-type GAD65 (lane 5). Western blotting of affinity purified M161T GAD65 was revealed with GAD6 (lane 6). Note the enrichment and/or formation during the purification of the 58-kDa C-terminal proteolytic fragment of GAD65 described in the text. Molecular-mass markers were present in lane 1.

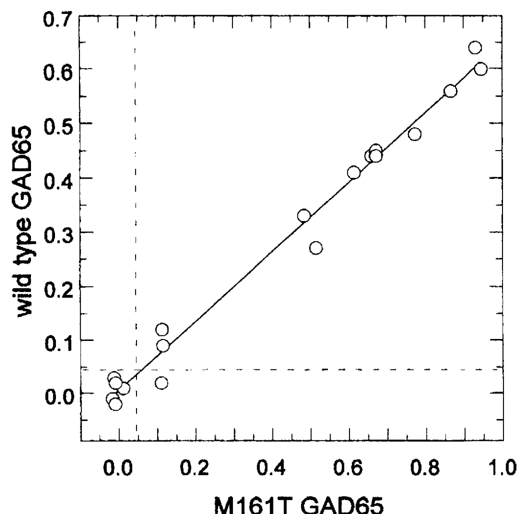

Figure 7 Correlation between the GAD Index determined by RBA with wild-type GAD65 and M161T GAD65

Sera from 18 type-1 diabetes patients and from 20 healthy controls were analysed. GAD Indices were calculated as described in the Materials and methods section. Dashed lines indicate the cut-off (mean+3 S.D. from the control GAD Index distribution) that separates positive from negative results.

The *M161T GAD65* gene (without the Trx extension) was amplified by PCR using pRH1GAD65(Trx) as the template and oligonucleotides 5′XbaI and 3′TOTAL as the primers. The amplification product was cut with *Xba*I and subcloned into the *Xba*I site of pSP64 poly(A) to yield pRH2GAD65, which was expressed in a reticulocyte lysate, producing [^{35}S]methionine–M161T GAD65. In parallel, wild-type [^{35}S]methionine–GAD65 was produced from pEX9 [20,26,27]. Labelled proteins were analysed by SDS/PAGE and fluorography, revealing a single 65-kDa band (Figure 6). M161T GAD65 and GAD65 yielded identical results in this experiment, which indicates that the changes introduced in the genetic message of M161T GAD65 were neutral in terms of the performance in the translation *in vitro*.

To assess if the Met-161 → Thr substitution disrupts critical conformational epitopes, GADA in serum samples were determined by the RBA reference method using *in vitro* translated GAD65 and M161T GAD65. Sera of 18 patients, with known index of GADA, and 20 normal sera were tested. As shown in Figure 7, the correlation of the GAD Index obtained with the two antigens was excellent ($r^2 = 0.985$). Interestingly, M161T GAD65 showed a moderate but significantly better performance than wild-type GAD65 in the assay. The improvement was illustrated by an increase in the GAD Index value, which in turn yielded a regression slope significantly different from unity (Figure 7; $b = 0.64 \pm 0.04$, 95% confidence interval). This result clearly demonstrated that the changes introduced in the sequence of GAD65 did not adversely affect the immunochemical properties of the antigen. On the contrary, in terms of the RBA, they might have had a moderate positive effect. A test with a much larger group of patients would be needed to definitively prove the improved behaviour of the new variant.

Heterogeneity in the expression of Trx–GAD65

We have reported previously that a significant fraction of Trx–GAD65, mainly in the inclusion bodies but also in soluble form, was expressed as a shorter, 48-kDa version of the enzyme. Amino acid sequencing and genetic-engineering experiments demonstrated that a second translation was being initiated from AUG at position 348 [19]. The production of this contaminant fragment was much higher at

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37 °C than at 30 °C. Nevertheless, even at 30 °C it lowered the yield of pure Trx–GAD65 and complicated the purification. In addition, it would be desirable to grow the cells at 37 °C, a temperature that improves the yield of GAD65. These considerations prompted us to introduce the Met-161 → Thr change reported here. As shown in Figure 8 (lane 4), the 48-kDa band was eliminated in the Western blot of lysates from cells expressing Trx–M161T GAD65.

During the expression of both Trx–GAD65 and Trx–M161T GAD65 an additional 58-kDa GAD65-immunoreactive fragment was produced that co-purified with the 77-kDa Trx–GAD65 through the As-matrix and the SEC column (Figure 8, lane 6). To establish the identity of this shorter component, electrophoretically separated bands were blotted on to PVDF membranes and subjected to 10 cycles of Edman degradation. The results (KAACACDQKP) indicated that the 58-kDa polypeptide was GAD65 starting at position 70. This position was identified previously as a hot proteolytic spot for GAD65 in eukaryotic cells [17]. In addition, proteolysis *in vitro* and *in vivo* at position 70 was reported to render soluble the membrane-bound GAD65 [17]. Thus this fragment is the result of the proteolysis, *in vivo* and/or during the purification of the enzyme.

Since the 58-kDa fragment does not contain the Trx moiety responsible for high-affinity binding to the As-matrix, the co-purification with Trx–GAD65 suggests that heterodimers of the 73- and 58-kDa subunits can be formed during expression in *E. coli*. Immunoprecipitation of purified Trx–M161T GAD65 with polyclonal anti-Trx antibody, followed by SDS/PAGE and Western blotting with GAD6, the monoclonal antibody against the C-terminal portion of GAD65, revealed that Trx–M161T GAD65 and the 58-kDa fragment are associated in solution (results not shown). Since the protein purification was performed under reducing conditions, it is unlikely that a spurious disulphide bridge joined the two subunits together in the heterodimer. On the contrary, it is very likely that the same non-covalent forces that promote the formation of the native GAD65 homodimer [36] also promote the formation of the heterodimer. Two experimental results support this interpretation of the results: (i) pretreatment of the heterodimer with a large excess of dithiothreitol did not cause the two subunits to migrate as monomers in the SEC [19]; (ii) a similarly truncated 58-kDa N-terminal form of GAD65 produced in *E. coli* by Chu and Metzler was fully active and purified as a dimer [37]. Taken together, these results lead us to propose that the 58-kDa subunit folds into a native-like structure able to interact with the full-length native GAD65 moiety. It is worth noting that most of the relevant epitopes recognized by GADA are located in the C-terminal region of the molecule. Thus, the presence of the heterodimer formed by Trx–M161T GAD65 and the 58-kDa fragment should not interfere with the use of the antigen in GADA tests.

It has been reported that tolerization to GAD65 prevents insulitis and diabetes in NOD (non-obese diabetic) mice [38–40]. If these findings are applicable to humans, it would be possible to protect individuals at risk by treating them preventively with GAD65, and this in turn would result in a greatly increased demand of pharmaceutical-grade GAD65. Trx–M161T GAD65 was designed as a reagent for use *in vitro* and might not be suitable for use *in vivo* because of possible undesirable side effects caused by the Trx moiety. Yet, it should be possible to produce M161T GAD65 from Trx–M161T GAD65 by specific proteolysis at the enterokinase site located close to the N-terminus of M161T GAD65. This procedure would yield a M161T GAD65 with only a three-residue N-terminal extension (see Figure 4). If necessary, any adverse effect caused by this short extension may be readily eliminated by re-engineering the linker between the two moieties.

Conclusions

In summary, we report here the cloning and construction of a hybrid human–rat gene coding for a variant of human GAD65 with improved expression properties compared with the wild-type protein. Also, the nature of two major byproducts in the expression of this protein was established and one of them was eliminated by genetic engineering. The expression with a good yield of properly folded, active and immunochemically competent GAD65 in *E. coli* should facilitate the production of this antigen in large amounts for massive application of immunochemical tests for GADA in populations at risk for future development of insulin-dependence.

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