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Recombinant human preproinsulin

Expression, purification and reaction with insulin autoantibodies in sera from patients with insulin-dependent diabetes mellitus

Heike Berg [a], Michael Walter [b], Ludwig Mauch [a], Jochen Seissler [c] and Wolfgang Northemann [a]

[a] *Department of Molecular Biology, ELIAS Entwicklungslabor, D-79114 Freiburg, Germany,*
[b] *Institute of Genetics, University of Düsseldorf, D-40225 Düsseldorf, Germany, and*
[c] *Department of Internal Medicine I, University of Ulm, D-89081 Ulm, Germany*

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A novel prokaryotic expression vector pGEX-6T was designed for high-level expression of recombinant fusion protein with a histidine-hexapeptide and glutathione-*S*-transferase at its *N*-terminus and the recombinant human preproinsulin at its *C*-terminus. Efficiency of expression was investigated in the *Escherichia coli* strain CAG456. The synthesized protein was sequestered in an insoluble form in inclusion bodies and was purified to homogeneity by one-step affinity chromatography based on the specific complex formation of the histidine-hexapeptide and a chelating matrix which was charged with Ni^{2+} ions. The antigenic nature of the purified recombinant preproinsulin fusion protein was evaluated by ELISA screening for insulin autoantibodies in selected sera from patients with recent-onset type 1 (insulin-dependent) diabetes mellitus classified by the existence of additional autoantibodies reactive against glutamic acid decarboxylase. 14% of the tested sera ($n = 43$) contained insulin autoantibodies which strongly recognized the recombinant human preproinsulin. Comparable measurements with both recombinant human preproinsulin and mature insulin suggested that the observed autoantigenicity of preproinsulin was mediated by the C-peptide or/and signal peptide.

Key words: Prokaryotic expression; Recombinant preproinsulin; Insulin autoantibody; Insulin-dependent diabetes mellitus; Chromatography, affinity

Introduction

Many eukaryotic proteins that are of interest for basic research or for medical applications such as diagnostic assays are often not available in the desired amount and purity from natural sources. Therefore, cloning and expression of foreign genes in various biological systems such as bacterial, yeast and mammalian cells are common

Correspondence to: W. Northemann, Department of Molecular Biology, ELIAS Entwicklungslabor, Obere Hardtstrasse 18, D-79114 Freiburg, Germany. Tel.: +49-761-47805-58; Fax: +49-761-47805-20.

Abbreviations: GAD_{65}, 65 kDa glutamic acid decarboxylase; GST, glutathione-*S*-transferase; IAA, insulin autoantibody; IDDM, insulin-dependent diabetes mellitus; IPTG, isopropylthiogalactoside; PBS, phosphate-buffered saline; PPI, preproinsulin.

procedures providing a ready source of these proteins. In the present paper we describe cDNA cloning and expression of recombinant human preproinsulin in *Escherichia coli* with respect to two objectives. First, we established satisfactory synthesis and purification of recombinant human preproinsulin as a fusion protein using the novel prokaryotic expression system pGEX-6T followed by a specific single-step purification based on affinity chromatography with a metal chelating matrix (Berthold et al., 1992). Second, we wished to evaluate recombinant human preproinsulin as a possible antigenic tool in ELISA measurements of insulin autoantibodies (IAA), e.g., in selected sera from patients with insulin-dependent diabetes mellitus (IDDM) which also contained autoantibodies directed against the 65 kDa glutamic acid decarboxylase (GAD_{65}).

Type 1 (insulin-dependent) diabetes mellitus is one of most severe and common autoimmune diseases characterized by irreversible and selective destruction of insulin-producing β cells in the pancreas and circulating islet specific autoantibodies which are present for years before clinical onset of the disease (for review see Castano and Eisenbarth, 1990; Boitard and Bach, 1991; Harrison, 1992). The detection of these autoantibodies in non-diabetic individuals indicates risk for IDDM and prediabetic insulitis. The anti-GAD_{65} autoantibodies are one of the earliest detectable islet-cell autoantibodies (Baekkeskov et al., 1982, 1990). While the anti-GAD_{65} autoantibody is currently the best studied of the islet-specific autoantibodies and serves as the most relevant serological marker for ongoing β cell destruction, the generation of insulin autoantibodies (IAA) alone confers relatively little risk for IDDM development.

Material and methods

Isolation of human preproinsulin cDNA

Approximately 1.5×10^6 clones of a human pancreatic carcinoma cDNA library in λ-gt11 (Clontech) were screened with a synthetic oligonucleotide corresponding to the cDNA sequence of human insulin (Goedel et al., 1980). 14 positive clones were obtained. Corresponding cDNAs were isolated from the λ clones, subcloned into plasmid Bluescript SK(f-) (Stratagene) and characterized by sequence analysis. The clone pPPI.3 carried the longest insert with 541 bp in length coding for the entire human preproinsulin including extended non-coding 5′ and 3′ regions and with a polyA stretch of 65 adenosines.

Expression of the preproinsulin fusion protein in Escherichia coli

Various *E. coli* strains transformed with the plasmid pPPI.304 were cultured overnight in LB medium containing 150 μg/ml ampicillin, diluted ten-fold with fresh prewarmed LB medium and incubated for 60 min prior to induction with 1 mM IPTG for 5 h. Bacteria in aliquots of 100 μl culture medium were sedimented, resuspended in sample buffer and applied to 10% polyacrylamide-SDS gel electrophoresis.

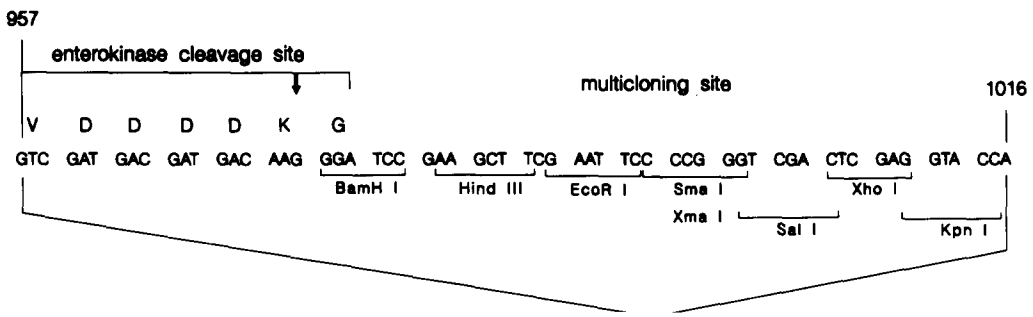
Immunoblotting

The proteins were separated by 10% polyacrylamide-SDS gel electrophoresis under reducing conditions and transferred to nitrocellulose filters (Amersham) using a trans-blot semi-dry electrophoretic transfer cell (Bio-Rad). The unoccupied protein-binding sites on the filter were blocked with 5% non-fat dried milk in TBST buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The immobilized proteins were incubated for 2 h with a 500-fold dilution of anti-porcine insulin serum from guinea pig (Sigma) or sera from IDDM patients. The anti-porcine insulin serum was preabsorbed with 0.1 mg/ml crude extract derived from *E. coli* strain LE392 transformed with pGEX-6T. The bound antibodies were visualized either with anti-guinea pig immunoglobulins (Sigma) or anti-human immunoglobulins (Promega) conjugated with alkaline phosphatase, respectively.

Purification of the recombinant preproinsulin fusion protein

E. coli strain CAG456 (Snyder et al., 1987) transformed with pPPI.304 was cultured in 100 ml LB medium and induced with 1 mM IPTG for 5 h. The cells were sedimented, resuspended in 20 ml PBS and treated with 1.0 mg/ml lysozyme (Sigma) and 1 mM phenylmethylsulfonyl fluoride

(Sigma) for 20 min at 0°C followed by incubation with 1% Triton X-100 (Sigma) for 10 min at 0°C. The cells were lysed by sonication with two pulses for 20 s each at about 100 W at 0°C. The soluble and insoluble cell fractions were separated by centrifugation of the cell homogenate at 8000 × *g* and 4°C for 5 min. The pellet containing the insoluble recombinant preproinsulin fusion protein was dissolved in 50 ml 6 M guanidinium-hydrochloride in Tris/acetate buffer (50 mM Tris/acetate, pH 7.8, 0.5 M NaCl) and directly applied onto a 10 ml chelating Sepharose FF column (Pharmacia) which was charged with nickel ions according to the producer's standard protocol (Pharmacia). The column was developed with a pH step gradient of pH 6.0, pH 5.5., pH 5.0 and pH 4.0 as described previously (Berthold et al., 1992). The recombinant protein eluted at pH 4.0 was dialysed against 6 M guanidinium-hydrochloride in Tris/acetate buffer, pH 7.8, without NaCl and treated with 20 mg/ml sodium sulfite, 10 mg/ml sodium tetrathionate and 1 mM EDTA for oxidative sulfitolysis for 6 h at room temperature (Patrick and Lagu, 1992). After subsequent dialysis against 2 M urea in 20 mM Tris/acetate, pH 8.0, and PBS at room temperature the preproinsulin fusion protein was concentrated and stored at −20°C. The recombinant



GST was expressed by pGEX-6T and purified with the same protocol.

ELISA screening for anti-insulin autoantibodies

The antigens were diluted in phosphate-buffered saline (PBS) and applied to microtiter plates at concentrations of 1 μg/ml. The coating was performed with 120 μl/well for 16 h at 4°C. The plates were blocked with PBS containing 0.5% bovine serum albumin (BSA), 1% lactose and 0.02% sodium azide, dried and stored in sealed bags until use at 4°C. The ELISA procedure was performed with 100 μl aliquots of serum from each patient which were diluted 100-fold with PBS supplemented with 0.5% BSA and 0.05% Tween 20. Samples were incubated in the plate for 30 min at room temperature. To prevent nonspecific immunoreaction the diluted sera were pretreated with a 0.1 mg/ml extract from *E. coli* strain LE392 transformed with pGEX-6T. After washing the plates, bound antibodies were visualized with rabbit anti-human IgG conjugated with horseradish peroxidase for 15 min at room temperature followed by a color reaction with *o*-phenylenediamine. The extinction was measured bichromatically at 492 nm with a 640 nm reference. The concentrations of IAA expressed in arbitrary units (U/ml) were calculated from a standard curve. The standard used in this immunoassay was taken from a commercially available insulin antibody ELISA kit (SYNELISA, ELIAS Medizintechnik) and comprised an isolated and defined immunoglobulin fraction specifically directed against highly purified human mature insulin. The interassay variance was less than 10% at all analyte levels. All measurements were undertaken with an automatic ELISA reader (SLT Labinstruments) using commercially available SYNELISA software (ELIAS Medizintechnik).

Autoantibodies

All sera from autoimmune patients with recent-onset IDDM tested in this study were positively assayed for anti-GAD_{65} autoantibodies by immunoprecipitation as described previously (Seissler et al., 1992). The antigen sample for screening the sera of patients was metabolically labeled recombinant full-length human GAD_{65} which was expressed in infected *Spodoptera frugiperda* (Sf9) insect cells mediated by recombinant baculovirus *Autographa californica* (L. Mauch, unpublished results).

Results

Construction of vector pGEX-6T

The initial expression vector pGEX-3T (Frorath et al., 1992) was modified in order to insert a

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                |<-- GST
MSPIHHHHHH LLGYWKIKGL VQPTRLLLEY LEEKYEEHLY ERDEGDKWRK    50

KKFELGLEFP NLPYYIDGDV KLTQSMAIIR YIADKHNMLG GCPKERAEIS   100

MLEGAVLDIR YGVSRIAYSK DFETLKVDFL SKLPEMLKMF EDRLCHKTYL   150

NGDHVTHPDF MLYDALDVVL YMDPMCLDAF PKLVCFKKRI EAIPQIDKYL   200

                     GST -->|        |<-- preproinsulin
KSSKYIAWPL QGWQATFGGG DHPPKSVDDD DKGALWMRLL PLLALLALWG   250

PDPAAAFVNQ HLCGSHLVEA LYLVCGERGF FYTPKTRREA EDLQVGQVEL   300

                                          -->|
GGGPGAGSLQ PLALEGSLQK RGIVEQCCTS ICSLYQLENY CN           342
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Fig. 2. Amino acid sequence of the preproinsulin fusion protein. The amino acid sequence was deduced from the analysed cDNA sequence of the GST fusion gene coding for a fusion protein with the GST (aa no. 13–224) at its N-terminus and human preproinsulin (aa no. 234–342) at its C-terminus as indicated. Both the histidine-hexapeptide and the enterokinase recognition sequence are underlined.

coding sequence for histidine-hexapeptide (H_{1-6}) and to replace the thrombin cleavage site by a corresponding recognition sequence (VDDD-DKG) for the site-specific enterokinase by corresponding oligonucleotide linker cloning (data not shown). The enterokinase cleavage site provided the option of cleaving the GST moiety of the purified fusion protein, if necessary (Hopp et al., 1988; Su et al., 1992). The new expression vector, pGEX-6T (Fig. 1), is 5023 bp long and directs the expression of recombinant fusion protein with glutathione-*S*-transferase (GST) combined with a histidine-hexapeptide as an affinity tail at its N-terminus. The highly efficient synthesis of recombinant protein is controlled by a *tac*-promoter (Amman et al., 1983) which is normally repressed

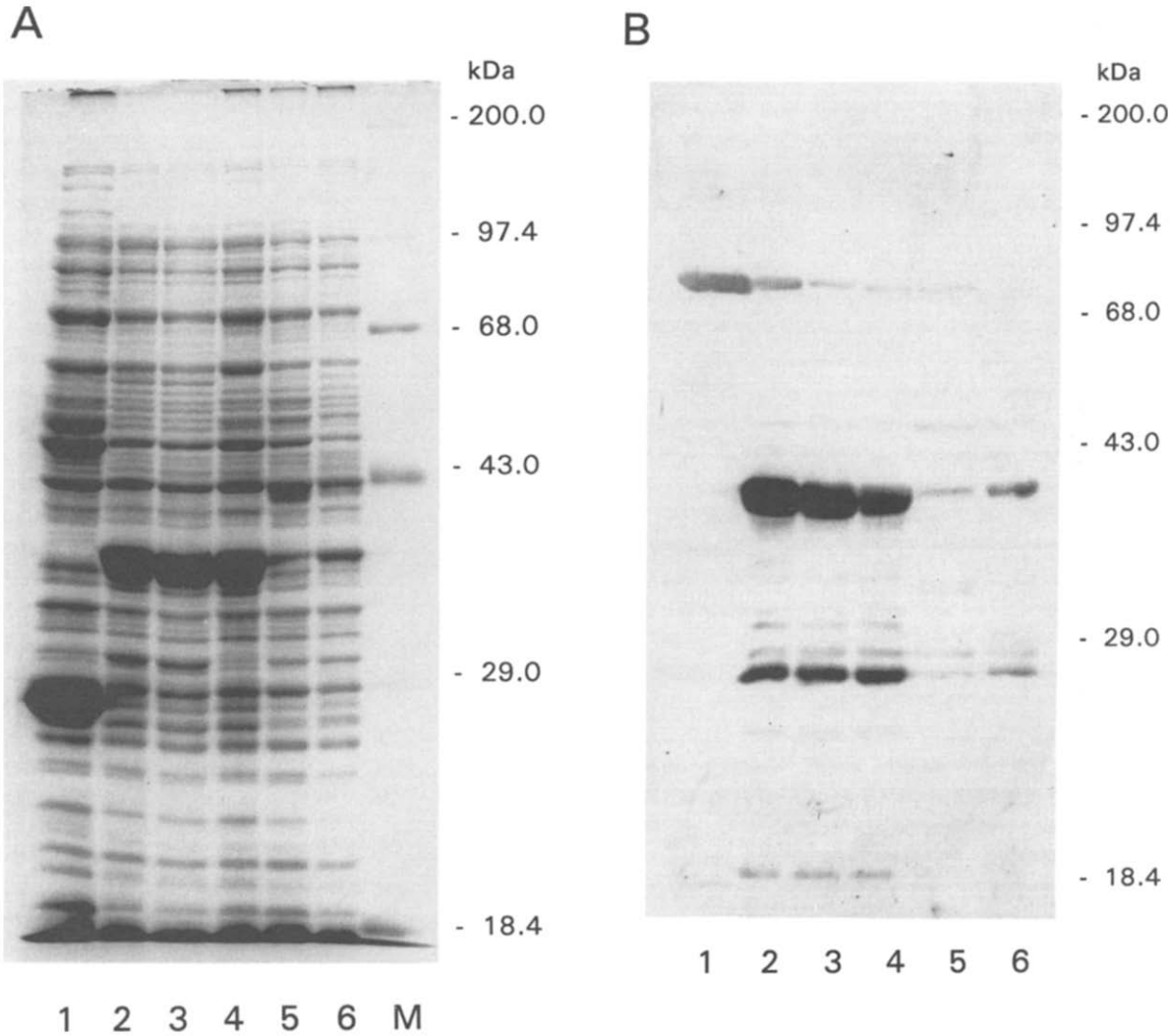

Fig. 3. Expression of recombinant preproinsulin fusion protein in various *E. coli* strains and their immunoblotting analysis. The *E. coli* strains CAG456 (lane 2), CAG440 (lane 3), GE196 (lane 4), LE392 (lane 5) and XL1-Blue (lane 6) were transformed with pPPI.304. Aliquots of 100 μl of bacterial cultures induced with 1 mM IPTG for 5 h were sedimented, resuspended in sample buffer and analyzed by 10% polyacrylamide-SDS gel electrophoresis. After separation the proteins were stained with Coomassie brilliant blue (A) or analyzed by immunoblotting using anti-porcine insulin serum (B). As a control, *E. coli* LE392 was transformed with pGEX-6T expressing the 26 kDa GST (lane 1). M = -Size markers.

by the *lac* repressor mediated by the *lacI*^{q} gene located on the plasmid until induction with IPTG.

Construction and expression of preproinsulin cDNA clone

In order to express the recombinant human preproinsulin as a GST fusion protein the full-length cDNA with 541 bp was isolated from the clone pPPI.3 in Bluescript, trimmed to remove the 5′ non-coding region including the initial codon for methionine by oligonucleotide linker replacement and inserted into the multicloning site of pGEX-6T resulting in the cDNA clone pPPI.304. The correct protein reading frame of the resulting GST fusion gene was established by DNA sequence analysis (Fig. 2).

Five *E. coli* strains, CAG456, CAG440, GE196, LE392, and XL1-Blue, were transformed with pPPI.304 and analysed for yield of the recombinant preproinsulin fusion protein. As a control the *E. coli* strain LE392 was transformed with pGEX-6T expressing the 26 kDa GST. After induction with 1 mM IPTG for 5 h, aliquots of the bacterial culture were analysed by polyacrylamide-SDS gel electrophoresis prior to staining of the proteins (Fig. 3*A*). Correct expression of the preproinsulin fusion protein with the expected molecular weight of 38 kDa was confirmed by immuno blotting analysis using commercially available anti-porcine insulin (Fig. 3*B*). The highest yield of recombinant protein was obtained with *E. coli* strains CAG456 and CAG440 (Fig. 3, lanes 2 and 3). The synthesised recombinant protein was gradually accumulated in CAG456 cells over a time period of 24 h (Fig. 4). Under optimal conditions the preproinsulin fusion protein accounted for up to 20% of total cellular proteins.

Purification of the recombinant preproinsulin fusion protein by affinity chromatography

The recombinant preproinsulin fusion protein was designed with an N-terminal histidine hexapeptide that permitted the simplified purification of recombinant proteins from bacterial lysates by affinity chromatography using a metal chelating matrix as previously described (Berthold et al., 1992). To examine whether the recombinant preproinsulin fusion protein directed by the

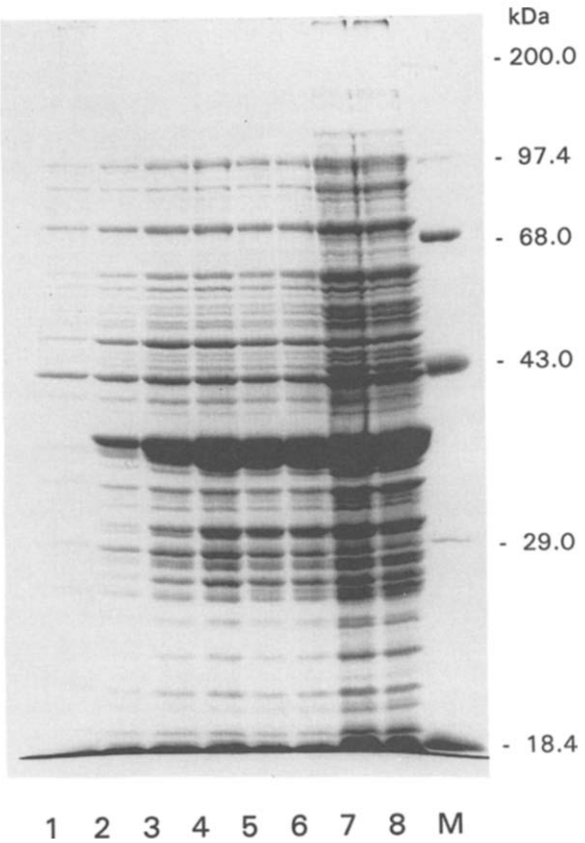

Fig. 4. Expression kinetics of recombinant human preproinsulin fusion protein. Cultures of *E. coli* CAG456 transformed with pPPI.304 were induced with 1 mM IPTG for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 6 h (lane 5), 8 h (lane 6), 12 h (lane 7) and 24 h (lane 8). Bacterial proteins in 100 μl aliquots of culture fluid were separated by 10% polyacrylamide-SDS gel electrophoresis and stained. (M) = Size markers.

clone pPP.304 was synthesized as a soluble or insoluble protein, the transformed *E. coli* strain CAG456 was induced with IPTG and lysed by sonication. The cell homogenate was separated into soluble and insoluble cell fractions by centrifugation and analysed by gel electrophoresis (Fig. 5, lanes 1, 2 and 3). The synthesized preproinsulin fusion protein was mainly sequestered as an insoluble cell fraction in inclusion bodies (Fig. 5, lane 3). In order to purify the recombinant protein the inclusion bodies were solubilized in 6 M guanidinium-hydrochloride and applied to a chelating Sepharose column charged with Ni^{2+}

ions (Berthold et al., 1992). The recombinant protein was eluted gradually at pH 5.5 and 4.0 (Fig. 5, lanes 5 and 6). The eluate at pH 4.0 contained recombinant preproinsulin fusion protein in almost pure form. Following dialysis the low solubility of the recombinant preproinsulin fusion protein could induce precipitation and in order to prevent this the intermolecular and intramolecular disulfide bridges were permanently disrupted by reaction with sulfite and tetrathionate a technique known as oxidative sulfitolysis (Patrick and Lagu, 1992). With this technique the purification of preproinsulin fusion protein yielded about 1.2 mg soluble recombinant protein per 100 ml bacterial culture of transformed *E. coli*.

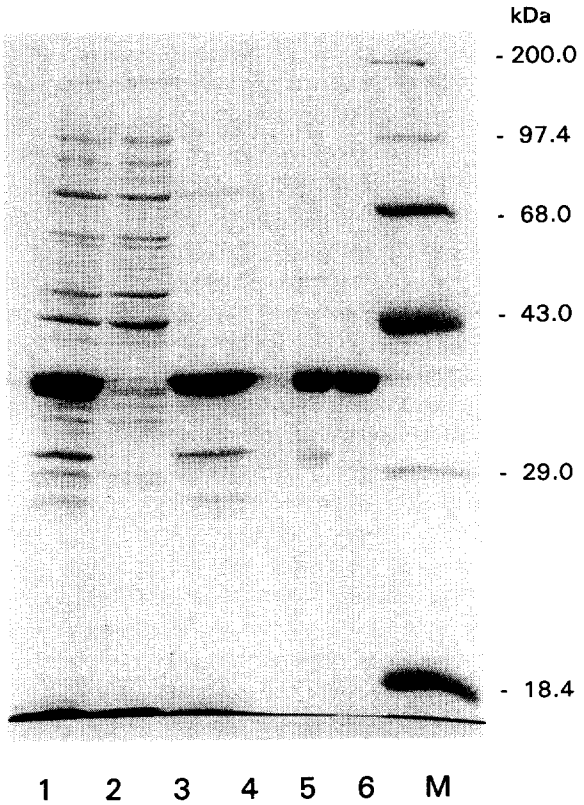

Fig. 5. Purification of recombinant human preproinsulin fusion protein. *E. coli* strain CAG456 transformed with pPPI.304 was cultured, induced with 1 mM IPTG for 5 h and fractionated to purify the recombinant fusion protein. 100 μl aliquots of culture fluid were removed from different cell fractions and from several steps of the purification by affinity chromatography on a metal chelating Sepharose FF column. *E. coli* lysate (lane 1), soluble cell fraction (lane 2), insoluble cell fraction (lane 3), fraction eluted at pH 5.5 (lane 4), at pH 5.0 (lane 5) and at pH 4.0 (lane 6). (M) = Size markers.

ELISA screening of IAA in sera of patients with IDDM using recombinant preproinsulin fusion protein

For the determination of IAA in sera from patients with IDDM a quantitative immunoassay (ELISA) was established with recombinant human preproinsulin fusion protein as an immobilized antigen target. In total 43 sera derived from patients with recent-onset IDDM and 39 sera from blood donors were compared (Fig. 6). The sera of the patients were positively selected for anti-GAD autoantibodies as a specific serum marker for IDDM measured by immunoprecipitation of metabolically labeled recombinant human GAD_{65} which was expressed by baculovirus in infected Sf9 cells (data not shown). With reference to the standard a cut-off was set at 10 U/ml between positive and negative sera. Six sera from patients with IDDM (14.0%) and one serum among blood donors (2.6%) reacted significantly with the recombinant preproinsulin fusion protein and exhibited IAA concentrations above the cut-off value (Fig. 6). The sera from the blood donors were randomly selected from a large pool. All sera used for the ELISA screening of IAA were pretreated with lysates of *E. coli* transformed with pGEX-6T which provided sufficient amounts of recombinant GST to prevent nonspecific reactions of the sera with the GST moiety of the preproinsulin fusion protein as well as with contaminating *E. coli* proteins (data not shown). No positive reactions were observed in ELISA measurements of the same pool of sera from IDDM patients based on recombinant mature human insulin as antigen target (data not shown).

Immunoblotting analysis with either purified recombinant preproinsulin fusion protein or GST as control were performed in order to confirm the positive ELISA measurements of the six sera from IDDM patients (Figs. 7*B*–7*G*) and the one serum from blood donors (Fig. 7*H*). As shown in Fig. 7 only five of the positive IDDM sera (11.6%) and the positive serum from the blood donors (2.6%) maintained their strong reactivity with the recombinant preproinsulin fusion protein. These data clearly indicated the existence of linear anti-

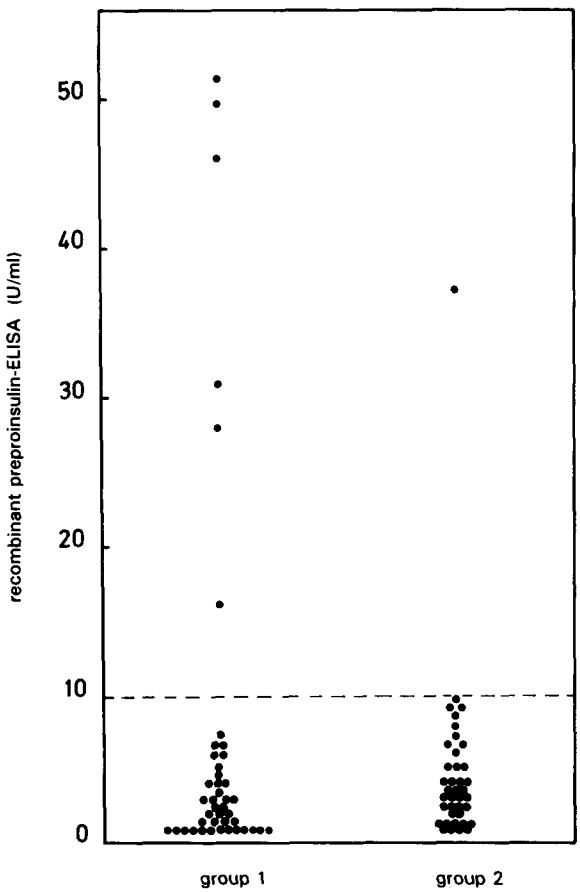

Fig. 6. ELISA screening of insulin autoantibodies in sera from patients with IDDM and blood donors using the recombinant preproinsulin fusion protein. Concentration of IAA illustrated in arbitrary units (U/ml) were assayed in 43 sera from patients with IDDM (group 1) and in 39 sera from blood donors (group 2) by an ELISA procedure using the preproinsulin fusion protein.

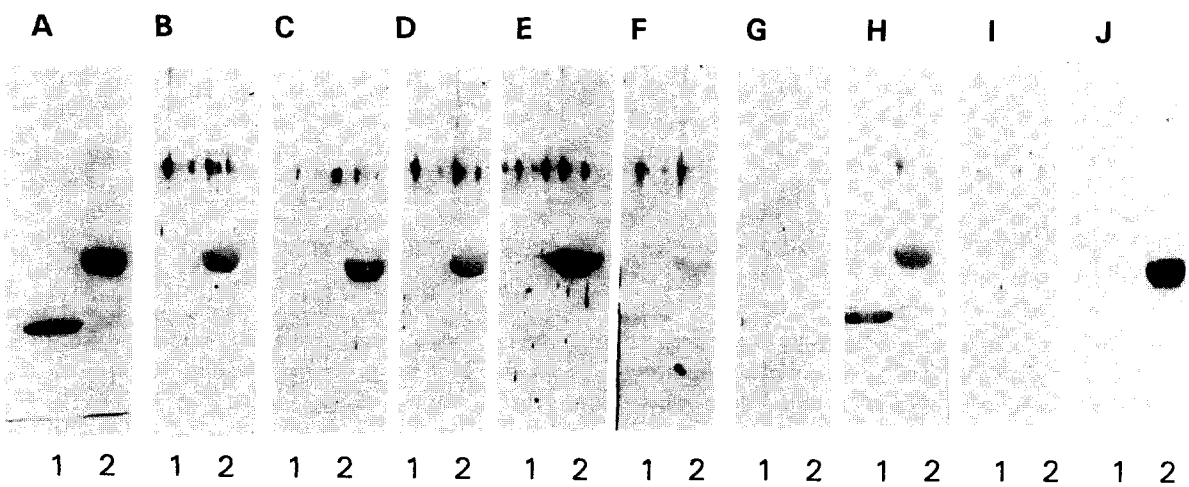

Fig. 7. Immunoblotting analysis of recombinant preproinsulin fusion protein using IAA positive IDDM sera. 2 μg of purified recombinant GST (lane 1) and preproinsulin fusion protein (lane 2) were separated under denaturing and reducing conditions by 10% polyacrylamide-SDS gel electrophoresis and blotted onto nitrocellulose filter. The separated proteins were stained with Coomassie brilliant blue (*A*) or analyzed by immunoblotting with sera from IDDM patients (*B*–*G*) and blood donors (*H*) which were positively assayed for IAA in an ELISA procedure as shown in Fig. 6. As a control the recombinant proteins were visualized by immunoblotting using a negative serum from blood donors (*I*) and an anti-porcine insulin serum from a guinea pig (*J*).

genic epitopes within the preproinsulin. One serum from the blood donors contained antibodies directed against recombinant GST. Therefore, some reactivity of the recombinant preproinsulin fusion protein may also be mediated non-specifically by the GST moiety (Fig. 7*H*). A negative serum from blood donors (Fig. 7*I*) and anti-porcine insulin serum (Fig. 7*J*) were included as negative and positive controls, respectively.

Discussion

The use of modern recombinant technology permits the synthesis and purification of human proteins in heterologous cell systems which are potenially useful for basic research or for many medical applications such as diagnostic assay systems. Many proteins such as human preproinsulin cannot be obtained from their natural sources in suitable quantities because of their low abundance or difficulty of purification by conventional methods from human tissue samples, organs or cell lines. One solution is the production of recombinant human proteins in heterologous prokaryotic cell systems such as *Escherichia coli* mediated by suitable and powerful expression vectors. In the present paper we describe the construction of a novel highly efficient prokaryotic expression vector as well as its use for the cloning, expression and purification of recombinant human preproinsulin. An additional aim of the present study was to investigate the use of recombinant human preproinsulin in ELISA screening for insulin autoantibodies (IAA).

In addition to autoantibodies directed against islet cell proteins (Bottazzo et al., 1980) and glutamic acid decarboxylase (Baekkeskov et al., 1982, 1990; Kaufman et al., 1992) the IAA are useful in the investigation diagnosis of IDDM (Palmer et al., 1983; Atkinson et al., 1986; Dean et al., 1986; Wilkin, 1990). In newly diagnosed IDDM patients the frequency of IAA is about 40% in children, but only 4% in adults (Karjalainen et al., 1989). The IAA may arise secondary to injury of the β cells with subsequent release of stored precursors of insulin having antigenic potential. Therefore, the use of preproinsulin representing the entire precursor molecule of insulin may be an appropriate technique for the measurement of IAA in the sera of patients with IDDM because it to also permits the detection of antibodies directed against the C-peptide or signal peptide.

A cDNA coding for the full-length human preproinsulin was isolated from a cDNA library derived from a pancreatic carcinoma cell line by screening with corresponding synthetic oligonucleotides based on the published cDNA sequence of human insulin (Goedel et al., 1980). For the production of recombinant human preproinsulin the cDNA was inserted into the newly designed prokaryotic vector pGEX-6T which provides high-level expression of a fusion protein with a histidine hexapeptide and glutathione-S-transferase (GST) at its N-terminus under the control of the *tac* promoter (Amman et al., 1983).

The pGEX-6T was designed with several objectives in mind. Firstly, we wished to express the human preproinsulin as a high molecular weight fusion protein with the carboxyl terminus of the GST from *Schistosoma japonicum* (Smith et al., 1988). The GST moiety is able to mediate protection of recombinant proteins against the proteolytic defense system of the host (Smith and Johnson, 1988) which is particularly common in the bacterial expression of small recombinant proteins such as the 12.5 kDa preproinsulin (data not shown). As shown in Figs. 3 and 4 the recombinant preproinsulin fusion protein was over-produced in the *E. coli* after induction with 1 mM IPTG and was intracellularly stable for up to 24 h. Secondly, we introduced a recognition sequence for the site-specific enterokinase (Su et al., 1992; Hopp et al., 1988) providing the opportunity, if necessary to cleave the GST moiety from the recombinant protein. This might be advantageous in certain situations, although it was not essential in the case of the preproinsulin used as antigen in the ELISA measurements. Thirdly, we fused a histidine hexapeptide to the N-terminus of the recombinant preproinsulin fusion protein to permit single-step purification by affinity chromatography using a metal chelating Sepharose charged with Ni^{2+} ions (Berthold et al., 1992). In general, high-level expression often leads to the intracellular accumulation of recombinant proteins in the form of insoluble inclusion

bodies. The inclusion bodies are only soluble in detergents, strong chaotropic reagents such as guanidinium hydrochloride, and in urea. These agents normally destroy the natural conformation of the recombinant proteins, and therefore prevent purification by conventional chromatography based on interactions with biologically active ligands. The fusion to a histidine hexapeptide permitted the ready purification of recombinant preproinsulin to near homogeneity after solubilizing the inclusion bodies with guanidinium-hydrochloride followed by affinity chromatography based on retention on a Ni^{2+}-Sepharose column and elution by a pH step gradient. Subsequent sulfitolysis (Patrick and Lagu, 1992) is suitable for the purification of recombinant proteins with either low solubility or high hydrophobicity by preventing precipitation after dialysis. Fourthly, we evaluated the most efficient host/vector system for pGEX-6T. The recombinant human preproinsulin fusion protein was preferentially produced at high levels in the *E. coli* strains CAG440 and CAG456 (Snyder et al., 1987). Because the pGEX-6T carries the gene coding for the *E. coli lac* repressor regulation of the expression of recombinant protein is independent on *lac* repressor synthesis by the host.

In order to examine the autoantigenic property of the bacterially expressed preproinsulin we analysed 43 selected sera from patients with recently diagnosed IDDM aged between 18 and 30 years. All sera were positively assayed for autoantibodies directed against GAD_{65} (Seissler et al., 1992). Such autoantibodies are commonly believed to be one of the major predictive serum markers for IDDM (Baekkeskov et al., 1982, 1990; Kaufman et al., 1992). The ELISA procedure identified six patients (14%) with autoantibodies able to react strongly with recombinant preproinsulin fusion protein. The frequency of IAA in sera from IDDM patients measured with the recombinant ELISA correlated with previously described results (Karjalainen et al., 1989; Wilkin, 1990; Greenbaum et al., 1992). Only five of the IAA positive sera (11.6%) maintained their reaction with recombinant preproinsulin in immunoblotting analysis confirmes the existence of linear antigenic epitopes within the preproinsulin. Bacterially expressed proteins normally lack any secondary structure or post-translational modifications. Interestingly, comparable studies of the same pool of IDDM sera were uniformly negative when recombinant mature human insulin was used as the antigen (data not shown).

In conclusion, the pGEX-6T construct represents a suitable cloning vector for high-level production of recombinant proteins as illustrated here for human preproinsulin. The comparable measurements of IAA with either recombinant preproinsulin or mature insulin suggest that IAA in IDDM sera assayed by this recombinant ELISA in IDDM could be directed against C-peptide or/and signal peptide. Future investigations should focus on the mapping of autoantigenic epitope(s) within human preproinsulin with a view to verifying the autoantigenicity of the C-peptide or signal peptide and the generation of IAA in the pathogenesis of IDDM.

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