

Characterisation of GLUT-2 and ICA12/SOX13 antibody reactivity in type 1 diabetes patients

Mitrea Cristina Mirela
Clinical University Hospital "N.Malaxa"

Host Laboratory: Laboratory of Molecular Genetics – Hospital San Raffaele, Milano, ITALY
Supervisors: Prof. Pierangelo BONINI, Prof. Maurizio FERRARI, Dr. Vito LAMPASONA

In this article

Acquired Technical Experience:

- Polymerase chain reaction
- Cloning in plasmid vectors
- Plasmid DNA extraction and restriction analysis
- *In vitro* transcription and translation
- Immunoprecipitation assay
- SDS-PAGE
- Western blot assay

Background

Type 1 diabetes is a chronic disorder due to the destruction of insulin producing pancreatic islet beta cells by the patient immune system. Diabetes autoimmunity is characterised by the presence of circulating autoantibodies to islet beta cell antigens. Prediction of type 1 diabetes is based upon the measurement of these autoantibodies and the identification and cloning of autoantigens is therefore important for prediction and eventual prevention of the disease. Identified autoantigens in type 1 diabetes include GAD, the protein-tyrosine phosphatase IA-2 and insulin. However, there exists evidence that several patients, especially those with slowly developing autoimmunity, have antibodies to still unidentified autoantigens.

Several proteins have been suggested to be target of these autoantibodies, however the majority of these putative targets have been difficult to confirm as true autoantigens in experienced laboratories. The major drawback in numerous studies apparently relates to the insufficient sensitivity and specificity of autoantibody tests based either on ELISA or western blot assays and the use of recombinant autoantigens expressed in bacteria. Autoantibodies are frequently low in titre compared to antibody responses to pathogens and recognise mostly conformational epitopes. Immobilisation of antigens on plates or nitrocellulose membranes easily results in the loss of conformational epitopes and in the exposure of cryptic epitopes that can be bound aspecifically by low affinity circulating antibodies. Moreover, bacterially expressed recombinant autoantigens are often poor autoantibody targets, as a con-

sequence of the frequent inability of bacteria to properly fold eukaryotic proteins, and also entails the risk of detecting aspecific antibody responses to contaminant bacterial proteins.

Rationale and specific aims

Before specific autoantibody routine testing is introduced in the clinical laboratory, putative autoantigens are in need of validation by sensitive and specific techniques, other than those usually applied to screening for novel protein target of autoantibodies. A consensus has been achieved in the type 1 diabetes research community that the most reliable assays are those based on the immunoprecipitation of radio-labelled recombinant antigens expressed either in an *in vitro* system, like rabbit reticulocytes, or *in vivo*, as a more cumbersome alternative, in a eukaryotic cell system, like recombinant baculovirus infected insect cell lines.

We decided to focus our attention on two different proposed type 1 diabetes autoantigens still lacking definitive confirmation. These are GLUT-2, a low affinity glucose transporter expressed at the cell surface of pancreatic islets beta cells, hepatocytes, small intestine and kidney epithelial cells, and ICA12/SOX13 an ubiquitous transcription factor highly expressed in the endocrine pancreas. Autoantibodies directed to GLUT-2 were initially reported in 1990 by JH Johnson et al.(1). In this study an inhibitory activity on glucose uptake by cultured rat pancreatic islets, but not rat hepatocytes

or human erythrocytes, was observed after incubation with purified IgG immunoglobulins from type 1 diabetes patients. The same group later identified the target of these antibodies as the GLUT-2, as indicated by selective inhibition of glucose uptake in GLUT-2 transfected cell line after incubation with patients' sera (2). The studies by another group is also supportive of this observation and reports the presence of autoantibodies to GLUT-2 detectable in western blot (3). The identification of a type 1 diabetes autoantigen named ICA12 was initially reported also in 1992 by D.U. Rabin et al. (4) upon screening of a pancreatic islets cDNA expression library with patients' sera. Autoantibodies to the ICA12 antigen were detected by western blot although at a relatively low frequency (5 out of 12 patients were found positive). No other study on ICA12 has reached publication until the recent identification of SOX13 a novel transcription factor of the HMG (high mobility group) family which share a very high degree of homology with ICA12.

The goal of our research project was therefore to develop assays for the measurement of autoantibodies to GLUT-2 and ICA12/SOX13 based on radio-labelled recombinant antigens expressed *in vitro* or *in vivo* in a eukaryotic system and to validate the presence of these two putative autoantigens in a cohort of type 1 diabetes patients.

Material and Methods

Sera: Sera from 100 newly diagnosed type 1 diabetes patients and 57 non diabetic age matched controls were used in the pilot test experiments. As a positive control serum for the GLUT2 antibody test a polyclonal rabbit anti-human GLUT2 antibody was used (Chemicon).

Cloning: The cDNA encoding the full length human GLUT-2 cDNA (GeneBank accession number J03810) and two overlapping cDNAs spanning the entire open reading frame of ICA12/SOX13 (GeneBank accession number AF098915) were obtained from purified human pan-

creatic islets. Total RNA was extracted from cells with RNeasy spin columns and reverse transcribed with SuperScript RNase H- reverse transcriptase (GIBCO) using an oligo-dT primer. Sequence specific PCR was then performed to amplify GLUT2 and ICA12/SOX13 cDNAs. These were then analysed by agarose gel electrophoresis, gel purified with AgarAce (Promega) and ligated directly into the pGEM-T-easy plasmid vector (Promega).

The ligation reaction was transformed into competent *E. Colii* cells of the X.L. Blue MRF' strain (Stratagene) and plated on agar plate containing ampicillin as selective agent. Several bacterial clones were subsequently grown in LB medium and plasmid DNA extracted with Quantum-prep spin columns (Biorad). Clones containing cDNA were identified by DNA restriction analysis

The GLUT2 cDNA was re-amplified with appropriate primers for subcloning into the pSPUTK plasmid vector. This vector contains an optimised leader sequence downstream of the SP6 phage promoter and allows the efficient transcription and translation of cloned cDNAs *in vitro*.

The GLUT-2 cDNA was also cloned into the pIZT/V5-His (Invitrogen) and pFastBac (GIBCO) vectors for subsequent expression *in vivo* in insect cell lines and positive recombinant clones identified by restriction analysis. For the pFastBac experimental procedure recombinant baculovirus genomes, or bacmids, were generated upon transformation of identified pFastBac-GLUT2 clones into *E. Colii* of the DH10Bac strain. Selected GLUT2-bacmid clones were then grown in LB medium and bacmid DNA extracted with a modified alkaline lysis method. A full length cDNA encoding ICA12/SOX13 was obtained by cutting with the Sph 1 restriction enzyme and ligation of the two partially overlapping original cDNA clones.

Expression *in vitro*: The plasmid DNA of isolated clones was used for *in vitro* coupled transcription and translation in the presence ³⁵S-methionine (Amersham) with

ICA12 partial constructs

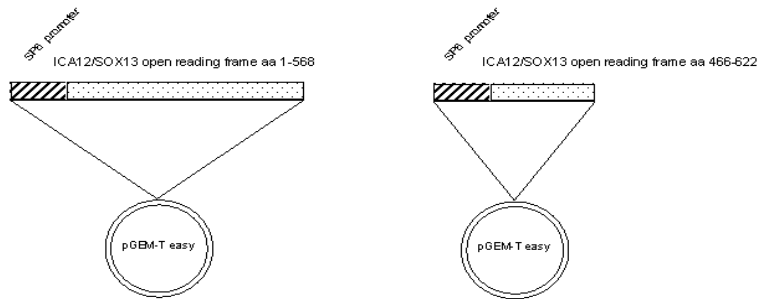


Figure no. 1

ICA12 partial constructs SDS-PAGE

Molecular weight markers
ICA12/SOX13 I portion pGEM-Teasy
ICA12/SOX13 II portion pGEM-Teasy

Figure no. 2

the TnT rabbit reticulocyte system (Promega). Recombinant radio-labelled proteins were then purified of unincorporated ^{35}S -methionine by size-exclusion chromatography on a NAP-5 column (Pharmacia), their incorporated radioactivity measured in a liquid scintillation beta counter (Kontron), and analysed by polyacrilamide gel electrophoresis under denaturing conditions followed by autoradiography to confirm expression of recombinant GLUT2 and ICA12/SOX13 proteins of the appropriate molecular weight.

Immunoprecipitation assays: The equivalent of 20,000 cpm of recombinant radio-labelled GLUT2 or ICA12/SOX13 antigens were incubated in duplicates in Tris-buffered saline, tween 1%, pH 7.4 (TBST) buffer with two microlitres of serum overnight at 4°C in 96-well deep well plates. Immune complexes were recovered by incubation with protein-A sepharose beads followed by centrifugation and washed 5 times with 750 microlitres of TBST. Protein-A sepharose beads were then transferred in 96-well opticount plates (Camberra Packard) and scintillation liquid added and recovered radioactivity measure in a TopCounter instrument.

Expression *in vivo*: SF9 insect cells were cultured in SF900 serum free medium (GIBCO) until early log phase. 0.5×10^6 cells were then plated in 6-well plates and transfected with 5 micrograms of recombinant pIZT/V5-His-GLUT2 plasmid using Insectin-Plus (Invitrogen) liposome mixture. After 4 hours incubation cells the transfection mixture was removed and replaced with fresh serum free medium. Cells from replicate wells were harvested at day 2, 3, 4 post transfection and lysed with HEPES pH7.4, Triton X-100 1% buffer containing a protease inhibitor cocktail (SIGMA). Efficiency of transfection was also monitored by fluorescence-microscopy, based on the co-expression of green fluorescent protein from the same vector. After day 4 for the pIZT/V5-His-GLUT2 experiment the antibiotic Zeocin was added to the culture medium to select for stable expression of GLUT2 from resistant cell lines. For the pFastBac experiment 0.5×10^6 cells were plated in 6-well

plates and transfected with 1 micrograms of GLUT2-bacmid DNA using CellFectin liposomes (GIBCO). After 4 hours incubation cells the transfection mixture was removed and replaced with fresh serum free medium. Cells from replicate wells were harvested at day 2, 3, 4 post transfection and lysed with HEPES pH7.4, Triton X-100 1% buffer containing a protease inhibitor cocktail. At day 4 the supernatant from transfected cells was collected, diluted 1 to 4 with fresh medium and added to SF9 cells in early LOG phase for amplification of eventual Baculovirus stock..

Western blot: monitoring of GLUT2 expression in transfected insect cell lines was performed by western blot analysis of collected cell lysates using the anti-GLUT2 polyclonal antibody and a chemiluminescent detection system.

Results

GLUT2 expression *in vitro* and immunoassay: GLUT2 expression was efficiently achieved *in vitro* from the pSPUTK vector,

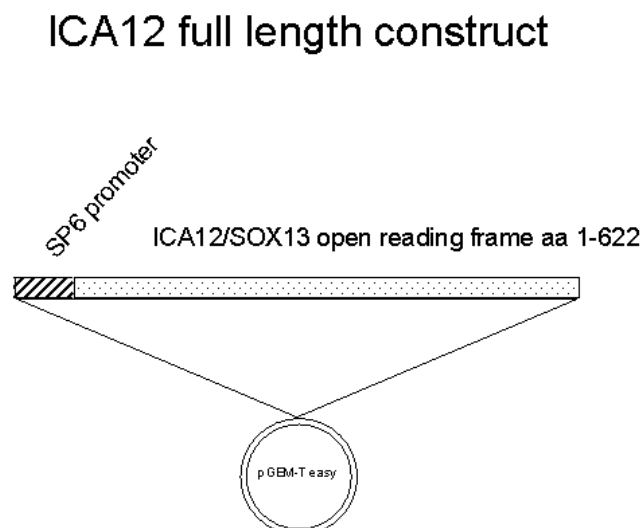


Figure no. 5

as demonstrated from incorporated radioactivity after coupled transcription and translation. However, upon autoradiography a protein product of apparent molecular size much larger than expected was observed. This recombinant protein was nev-

GLUT2 construct

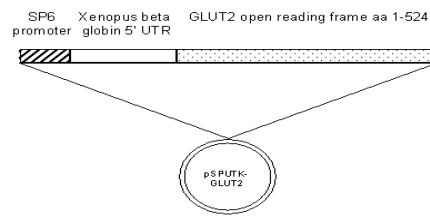


Figure no. 3

GLUT2 SDS-PAGE

Molecular weight markers
GLUT2 pSPUTK
GLUT2 pGEM8zf
GLUT2 pGEM-Teasy

Figure no. 4

ertheless immunoprecipitated by the polyclonal GLUT2 antibody. An immunoprecipitation assay based on this recombinant protein failed to show differences in binding of antibodies between patient and control sera.

GLUT2 expression *in vivo* and western blots: GLUT2 expression was tested in transfected insect cells using two different expression vectors. In neither case expression of a recombinant protein of the expected molecular weight could be observed using the polyclonal GLUT2 antibody.

ICA12/SOX13 expression *in vitro* and immunoassay: ICA12/SOX13 expression *in vitro* was achieved from both amplified and cloned open reading frames and the full length construct. The recombinant proteins observed upon autoradiography showed the expected molecular size. Binding to both ICA12/SOX13 constructs after immunoprecipitation was observed in a proportion of type 1 diabetes sera. A minority of control sera reacted with only one of the construct. Immunoprecipitation of the full length ICA12/SOX13 protein indicated presence of autoantibodies in a minority of type 1 diabetes patients.

Discussion

Cloning and expression of GLUT2 using two different approaches could not be satisfactorily reached. In one case expression *in vitro* of cloned GLUT2 cDNA resulted in a product of unexpected molecular size, although still recognised by a polyclonal anti-GLUT2 antibody. It is unclear at the moment whether this large protein is a multimeric form of GLUT2 which includes several GLUT2 proteins, possibly covalently bound together upon translation, or whether protein other than GLUT2, present in the translation reaction, are then cross-linked to the recombinant GLUT2. Lack of binding from type 1 diabetes sera in immunoprecipitation could therefore either be ascribed to poor antigenicity of the recombinant GLUT2 or simply absence of autoantibodies. In the *in vivo* expression systems GLUT2 expression could not be convincingly demonstrated nor stable expression GLUT2 in-

ICA12 full length SDS-PAGE

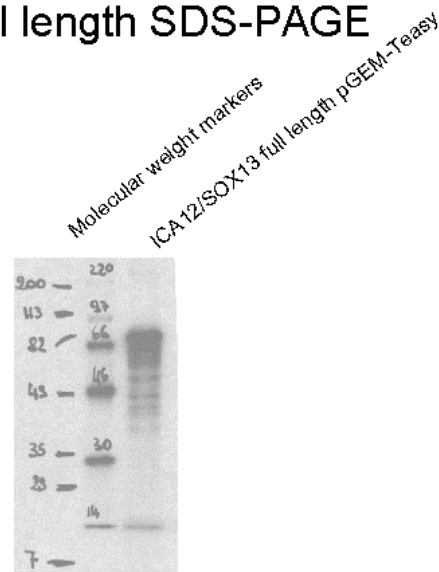


Figure no. 6

GLUT2 immunoprecipitation results

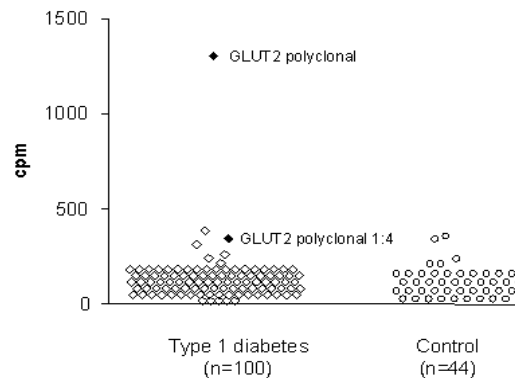


Figure no. 7

sect cell lines established. Lack of expression in this system is usually associated with inappropriate leader sequences in the recombinant cDNA and therefore ineffi-

cient translation in cells or to a secondary negative effect on cell growth or viability due to the recombinant protein in expressing cell. Both possibilities remains to be investigated.

ICA12/SOX13 expression *in vitro* proved more straightforward and preliminary experiments yielded two partial recombinant protein. Both these protein could be bound by antibodies present in sera from type 1 diabetes patients although with different backgrounds in the control sera group. Antibody testing with the full length ICA12/SOX13 clone now available in our laboratory indicated the presence of autoantibodies to ICA12/SOX13 in a minority of patients with type 1 diabetes. Whether this autoantibodies are associated with phenotypic differences in patients, like HLA antigens or other autoimmune pathologies, remains to be elucidated.

Acquired Technical Experience:

- Polymerase chain reaction
- Cloning in plasmid vectors
- Plasmid DNA extraction and restriction analysis
- *In vitro* transcription and translation
- Immunoprecipitation assay
- SDS-PAGE
- Western blot assay
- Insect cells culture and transfection

ICA12/SOX13 immunoprecipitation results

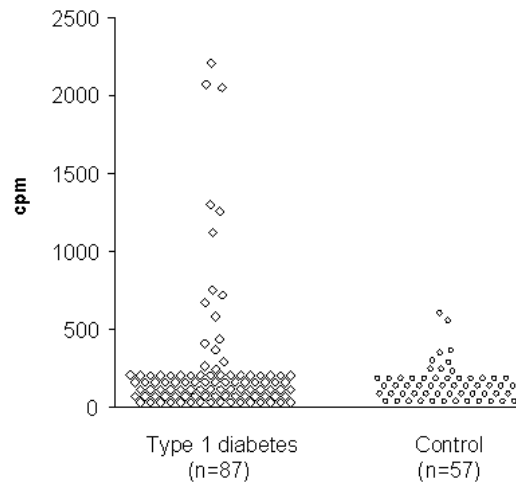


Figure no. 8