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Peptide mapping and characterisation of glycation patterns of the glima 38 antigen recognised by autoantibodies in Type I diabetic patients

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Abstract

Aims/hypothesis. Glima 38 is an *N*-glycated neuroendocrine membrane protein of M_r 38 000, which is recognised by autoantibodies in approximately 20 % of patients with Type I (insulin-dependent) diabetes mellitus. The aim of this study was to characterise the carbohydrate moiety and generate peptide maps of glima 38.
Methods. Sera of high immunoreactivity to glima 38 were used to isolate 35-S methionine-labelled protein from βTC-3 cells and a neuronal cell line GT1.7. Tunicamycin was used to inhibit *N*-glycation of glima 38 and define the core protein. The carbohydrate moiety was characterised for tunicamycin sensitivity, lectin binding and susceptibility to different endoglycosidases. The protein moiety was subjected to digestion by proteases to define peptide maps.
Results. The autoreactive epitopes in glima 38 recognised by Type I diabetic sera are conformational and independent of the carbohydrate moiety. Inhibition of *N*-glycation of glima 38 in vivo, shows a protein core of M_r 22 000 in both pancreatic β-(βTC3) and neuronal (GT1.7) cell lines. The carbohydrate moieties in the two cell types are distinct but contain a similar amount of terminal sialic acid residues and at least five oligosaccharide chains Glima 38 binds *Triticum vulgare* and *Ricinus communis I* lectins. Endoproteinase treatment of the M_r 22 000 core protein results in peptides of M_r 4500 and M_r 20 000 with Lys-C, and peptides of M_r 4 000 and M_r 11 000–12 000 with Glu-C/V8 and Asp-N proteases.
Conclusion/interpretation. The biochemical properties of glima 38 define it as a new autoantigen in Type I diabetes and provide a basis for its purification. [Diabetologia (2000) 43: 598–608]

Keywords Type I diabetes, immunology, autoantibodies, target autoantigen, 38 000 M_r autoantigen, glima 38, proteolytic cleavage, peptide mapping, lectin binding, deglycation.

Type I (insulin-dependent) diabetes mellitus is a chronic autoimmune disease, where the rapid onset is often preceded by a long prediabetic phase [1]. The gradual destruction of insulin-producing beta cells is accompanied by circulating autoantibodies to various islet cell antigens. Antibodies to islet cells

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Abbreviations: ConA, Concanavalin A from *Canavalia ensiformis*; GADA, antibodies to GAD65; glima 38; glycated islet cell membrane antigen of 38 000 M_r; IA-2A, antibodies to IA-2ic; IAA, insulin autoantibodies; ICA, islet cell antibodies; IgG; immunoglobulin G; LcH, lectil lentin from *Lens culinaris*, LEA, *Lycopersicon esculentum* lectin; MAA, *Maackia amurensis* lectin; NHS, normal human serum; PAS, protein A Sepharose; PFAP, particulate fraction, aqueous phase; PFDP; particulate fraction, detergent phase; PNA, *Arachis hypogaea* lectin; RCA-I, *Ricinus communis* lectin I; TBS, TRIS-buffered saline; TBST, TBS/0.01 % Tween 20; UEA, *Ulex europaeus* lectin; WGA, wheat germ agglutinin.

(ICA), made visible by immunofluorescence on frozen sections of human pancreas, are observed in 60–90 % of patients with newly diagnosed Type I diabetes and can react to one or more beta-cell antigens [2]. Thus, antibodies to glutamic acid decarboxylase, GAD65, [3] are present in 70–80 % [4, 5] and antibodies to a putative tyrosine phosphatase IA-2 [6–8] are present in 60–70 % of newly diagnosed Type I diabetic and prediabetic patients [5, 9]. Autoantibodies to both GAD65 and IA-2 immunoprecipitate their target antigen from lysates of islet cells in contrast to autoantibodies to insulin [10], which are present in 16–70 % of newly diagnosed Type I diabetic patients [2].

We have identified a third islet autoantigen, glima 38, which is immunoprecipitated by autoantibodies in sera from a subset of Type I diabetic patients [4]. Glima 38 is an amphiphilic N-Asp glycated beta-cell membrane protein, expressed in islets and in islet and neuronal cell lines [4]. Several other proteins of a relative molecular mass (M_r) of 38 000 have been implicated as autoantigens in Type I diabetes [11–15, 4]. Some of these molecules have been identified. A M_r 38 000 rat insulinoma protein was described to be the target of a $CD4^+$ T-cell clone derived from a Type I diabetic patient [11]. This protein was sequenced and named imogen 38 [15]. Imogen 38 is not N-Asp glycated, has a wider tissue distribution than glima 38 and does not seem to be a target of humoral immune response in Type I diabetes [15]. Antibodies to a second M_r 38 000 protein, the nuclear transcription factor jun-B, were detected in newly diagnosed Type I diabetic patients. In contrast to glima 38, jun-B is a non-glycated, soluble nuclear protein [13]. Thus both imogen 38 and jun B are distinct from glima 38 [4]. In an animal model of Type I diabetes, the *BB* rat, antibodies to yet another protein of M_r 38 000 have been described [14, 16].

The aim of this study was to test whether glima 38 is recognised by *BB*-rat autoantibodies to a 38 000 M_r protein and to characterise its glycation and proteolytic degradation patterns.

Subjects and methods

Antisera. Patients with Type I diabetes were from the Adult and Pediatric Diabetes Practices at the University of California San Francisco and the Third Medical Department, City Hospital Schwabing, Munich. Type I diabetes was diagnosed according to World Health Organisation criteria [17]. Diabetes was classified as Type I (insulin-dependent) on the basis of clinical presentation, including lean body habitus, lack of acanthosis nigricans, presence of diabetic ketoacidosis, low or absent serum insulin or C peptide and/or presence of islet cell autoantibodies. Sera were obtained after informed consent from 44 patients with Type I diabetes at clinical onset or within 6 months of diagnosis (mean age ± SD: 22.2 ± 14.6 years; range: 1–54 years; median 17 years). Sera of two first-degree relatives of Type I diabetic patients (Table 1: U9 and U122) were also analysed. This study was approved by the Committee on Human Research at the University of California San Francisco.

Table 1. Glima 38 antibody-positive subjects identified amongst 44 newly diagnosed Type I diabetic patients and first-degree relatives of Type I diabetic patients

Patient	Serum no.	sex	age (years)	GAD65 antibodies	IA-2ic antibodies	glima 38 antibodies
1	U38	f	6	neg	pos	pos
	U63		6.5	neg	pos	neg
2	U45	f	1	pos	pos	pos
3	U46	m	18	neg	pos	pos
4	U47	m	14	pos	pos	pos
	U86		14.6	neg	pos	pos
5	U53	f	26	pos	pos	pos
6	U55	m	10	pos	pos	pos
7	U56	m	42	pos	pos	pos
8	U70	m	8	pos	pos	pos
9	U76	m	N/A[a]	pos	pos	pos
	U77		N/A[a]	pos	pos	pos
	U80[b]		N/A[a]	pos	pos	pos
10	U87	f	2	pos	pos	pos
11[d]	U9	f	11.5	neg	neg	pos
	U40		12	pos	neg	pos
12[d]	U122[c]	f	3	neg	neg	pos

[a] N/A = Not available; [b] purified IgG from plasma collected during plasmapheresis of patient 9 [18] 2 weeks after drawing of serum U76. Serum U77 was drawn at the time of plasmapheresis. [c] Progression of Type I diabetes 2 years after serum was collected. [d] ICA/IAA positive first-degree relatives of Type I diabetic patients

Sera were screened for glima 38 antibodies, GAD65 antibodies (GADA) and IA-2ic antibodies (IA-2A) as described below. Follow-up sera were available for 4 of the glima 38 antibody positive subjects (Table 1: U38, U47, U76, U9). One of these patients (U76) underwent plasmapheresis and purified immunoglobulin G (IgG) from one treatment was used to prepare an immunoaffinity resin for glima 38 (Table 1: U80; [18]). The purified IgG fraction is positive for ICA, insulin autoantibodies IAA, GADA and IA-2A and has a concentration of IgG fivefold lower than in the patient's original serum sample (U76; data not shown).

We obtained five *BB*-rat sera positive and five *BB*-rat sera negative for antibodies to a rat membrane-bound islet cell-specific 38 000 M_r autoantigen [14, 16] from Dr. Yoon, University of Alberta, Calgary and tested for the presence of antibodies to glima 38 as described below.

Preparation of cell extracts enriched in glima 38. The murine pancreatic insulinoma βTC3 cell line [19], a gift from Dr D. Hanahan, University of California San Francisco, was used in all experiments except if otherwise indicated. We used GT1.7, a gonadotropin-releasing hormone secreting tumour cell line with a strong neuronal phenotype [20], a gift from Dr R. Weiner, University of California San Francisco, for the characterisation of glima 38 in cells of neuronal origin. The preparation of radiolabelled detergent phase purified membrane proteins was done as described previously [4] except that the Triton X-114 detergent phase [21] of the particulate fraction (PFDP) was subjected to an additional ultracentrifugation at 100 000 *g* to remove aggregates. The PFDP was precleared twice with normal human serum (NHS) before immunoprecipitation.

Analyses of antibodies to glima 38, GAD65 and IA-2. Aliquots of Triton X-114 detergent phase extracts (approximately 20 μl per immunoprecipitate) containing 4–5 × 10^6 cpm were precleared and ultracentrifuged as described above and incubated overnight at 4 °C with 12 μl test serum. Glima 38 antibody-positive and antibody-negative control sera were included in each experiment. Immunocomplexes were isolated using protein A Sepharose (PAS, human sera) or protein G Sepharose (rat sera [22]) (both from Pharmacia LKB Biotechnology, Piscataway, N.J., USA) and washed six times as described previously [4]. The washed PAS pellets were boiled in SDS-sample buffer (60 mmol/l TRIS-HCl, pH 6.8, 12.5 % glycerol, 80 mmol/l dithiothreitol, 5 % SDS, 0.01 % bromphenol blue) and analysed by SDS-PAGE using 12 % polyacrylamide gels followed by autoradiography using a PhosphorImager and the Image Quant Version 3.3 software (Molecular Dynamics, Sunnyvale, Calif., USA). Antibodies to GAD65 or IA-2 were analysed using radioligand binding assays [4, 23]. In each assay, sera were considered positive if the autoantigen was detected in SDS-PAGE analysis of the immunoprecipitate. In the 1995 GAD65 and IA-2 antibody workshop [24], the GAD65 antibody assay had a sensitivity of 88 % in Type I diabetic patients and a specificity of 98 %. The IA-2 antibody assay had a sensitivity of 79 % in Type I diabetic patients and a specificity of 97 %.

Generation of deglycated glima 38

Immunoprecipitates of glima 38 were prepared as described above and subjected to enzymatic deglycation according to the manufacturer's instructions. In each case enzymatic reactions were terminated by adding 1/3 volume of 4 × SDS-sample buffer and boiling. Samples were analysed by SDS-PAGE using 15 % polyacrylamide gels followed by autoradiography.

PNGase F. (New England Biolabs, Beverly, Mass., USA). Immunocomplexes isolated on PAS were eluted by boiling for 10 min in a 50 mmol/l sodium phosphate buffer, pH 7.5, containing 0.05 % SDS and 1 % β-mercaptoethanol. The PAS was removed by centrifugation and Nonidet P-40 (NP-40, from Calbiochem-Novabiochem, San Diego, Calif., USA) was added to a final concentration of 1 % before incubation with or without 5,000–10 000 U PNGase F in a final volume of 30 μl for 18 h at either 37 °C or 4 °C.

Neuraminidase. (Boehringer Mannheim, Indianapolis, Ind., USA). Immunocomplexes were eluted by boiling for 10 min in a 50 mmol/l sodium acetate buffer, pH 5.0, containing 0.2 % SDS and 1 % β-mercaptoethanol. After removal of PAS, supernatants were incubated with 10–50 mU neuraminidase in a final volume of 30 μl for 18 h at 37 °C.

O-Glycosidase. (Boehringer Mannheim). Immunocomplexes were eluted by boiling for 10 min in 50 mmol/l sodium phosphate buffer, pH 7.5, containing 0.1 % SDS and 0.7 % β-mercaptoethanol. After removal of PAS, Triton X-100 was added to a final concentration of 1 % and the supernatants were incubated with 0.5–2.5 mU O-glycosidase in a final volume of 30 μl for 18 h at 37 °C.

To inhibit *N*-glycation of glima 38 in vivo, tunicamycin (5–10 μg/ml) (Sigma Chemical Company, St. Louis, Mo., USA) was added to the medium during radiolabelling [26]. Glima 38 was isolated by immunoprecipitation and analysed by SDS-PAGE using 15 % polyacrylamide gels.

In vivo pulse-chase labelling. The synthesis and processing of glima 38 was analysed by pulse-chase-labelling. First βTC3-cells were incubated in DME-H21 methionine-free medium containing 1 % dialysed fetal calf serum (Gibco, Rockville, Md., USA) for 40 min and then with [^{35}S]-methionine (7.4 MBq) for 20 min followed by immediate replacement of the "pulse" medium with medium containing 0.2 mmol/l cold methionine ("chase" medium). Plates were harvested at different time points (0, 15, 30 min and 1, 2, 4, 6 h) and processed as described above. The PFDPs were prepared from each time point, immunoprecipitated with glima 38 antibody positive and negative sera and analysed by SDS-PAGE and fluorography.

Western blot analysis of glima 38. Immunoprecipitates of glima 38 were subjected to SDS-PAGE and blotted to a polyvinylidene diflouride (PVDF) membrane. The blots were blocked overnight in 10 % non-fat dry milk in TRIS buffered saline (TBS) containing 0.1 % Tween (TBST) and then incubated with glima 38 antibody positive sera (dilutions: 1:1–1:25 in TBST containing 5 % non-fat dry milk) for 1 h at room temperature. The blots were washed three times in TBST. Binding of antibodies was made visible using alkaline phosphatase or horseradish peroxidase conjugated anti-human IgG, and chemiluminescence (ECL reagent; Amersham, Arlington Heights, Ill., USA).

Peptide mapping of glima 38 by endoproteinase treatments

Immunoprecipitates of glima 38 and IA-2 were eluted from PAS and subjected to deglycation with PNGase F. Immunoprecipitates of IA-2 radiolabelled in vitro were processed in parallel to identify IA-2-derived peptides. Enzymatic reactions were terminated by addition of 1/3 volume of 4 × SDS-sample buffer and boiling. Peptides generated by enzymatic cleavage were separated on a 16 % TRIS/Tricine gel [26] and subjected to autoradiography.

Lys-C. (cleavage site: -Lys-↓-X- ; Boehringer Mannheim). One volume of 200 mmol/l ammonium bicarbonate buffer, pH 8.4, was added to the sample treated with PNGase F and incubation was continued with or without Lys-C (0.15–0.3 U in a final volume of 50 μl) for 8 h at 37 °C.

Asp-N. (cleavage site: -↓-Asp/Cys SO_3H-X- ; Boehringer Mannheim). One volume of 50 mmol/l sodium phosphate buffer, pH 8.0 was added to the sample treated with PNGase F and incubation with or without Asp-N (0.04–0.08 μg in a final volume of 50 μl) was continued for 3 h or 18 h at 37 °C.

Glu-C/V8. (cleavage site: -Glu-↓-X- ; Boehringer Mannheim). One volume of 50 mmol/l ammonium bicarbonate buffer pH 7.8 was added to the sample treated with PNGase F and incubation with or without Glu-C/V8 (1–2 μg in a final volume of 50 μl) was continued for 3 h or 18 h at 25 °C.

Lectin affinity chromatography of glima 38. The following lectins were obtained from EY Laboratories, San Mateo, Calif., USA), and tested for their ability to bind the glycated form of glima 38: *Canavalia ensiformis* (ConA), *Lens culinaris* (LcH), *Ricinus communis I* (RCA-I), *Triticum vulgare* (WGA), *Arachis hypogaea* (PNA), *Ulex europaeus I* (UEA), *Lycopersicon esculentum* (LEA) and *Maackia amurensis* (MAA). Immobilised lectin columns (500 μl) were equilibrated with 10 gel volumes of 10 mmol/l phosphate buffered saline (PBS), pH 7.45 (RCA-I, WGA, UEA-I, PNA, LEA, and MAA) or 50 mmol/l

TBS, pH 7.0–7.2 (ConA and LcH). Aliquots of precleared PFDP from βTC3 cells were applied to the columns and incubated for 15 min. Columns were washed with buffers (see above) to remove unbound material. Bound material was eluted using 2–3 gel volumes of buffer containing the appropriate competing carbohydrate according to the manufacturers instructions. After elution, the columns were washed with 10 gel volumes of 1.0–1.4 mol/l NaCl in distilled water and re-equilibrated by washing with about 50 gel volumes of storage buffer (PBS or TBS containing 0.1 % sodium azide). Flow-through, wash fractions and eluted fractions were immunoprecipitated with a glima 38 antibody positive serum and analysed by SDS-PAGE and autoradiography.

Immunoaffinity chromatography of glima 38. Purified IgG from plasma of the glima 38 antibody-positive Type I diabetic patient (U80) was coupled to protein A Sepharose beads to generate an affinity matrix for the purification of glima 38. Immunoglobulin G was incubated with preswollen PAS (6 mg IgG per ml swollen gel) in PBS at 4 °C for 1 h on a rotor. The beads were washed twice with 10 bead volumes of 200 mmol/l Na^+-borate pH 9.0. Cross-linking was done by incubation in 20 mmol/l dimethylpimelimidate for 30 min at 25 °C on a rotor. The reaction was stopped by adding 10 bead volumes of 200 mmol/l ethanolamine, pH 8.0 and mixing gently for 2 h at 25 °C. The effectiveness of coupling was analysed by SDS-PAGE using aliquots before and after the addition of dimethylpimelimidate. Beads used for immunoaffinity chromatography had a coupling effectiveness of 95 % or more. Before immunoaffinity chromatography, the beads were prewashed sequentially with the following solutions: 100 mmol/l $NaHCO_3$, pH 8.0; 1 mol/l NaCl; 100 mmol/l acetic acid, pH 4.0; and 1 mol/l NaCl. Finally the beads were washed and resuspended in 10 mmol/l HEPES, pH 7.4, containing 150 mmol/l NaCl, 10 mmol/l benzamidine, 5 mmol/l EDTA, 100 mg/l BSA, 1 % Triton X-114.

Aliquots of radio labelled PFDP from βTC3 cells were precleared by a batchwise incubation with beads prepared as described above but using IgG from normal human serum. The precleared extract was incubated at 4 °C for with U80 IgG immunoaffinity beads (170×10^6 cpm/ml packed beads) by gentle rotation. Maximum binding of glima 38 to the immunoaffinity resin was achieved after a 1-h incubation (as determined in pilot experiments). The beads were then poured on a column. The flow-through was collected, followed by ten washes (2-bead volumes each) before elution of bound material. After optimising of the elution conditions (data not shown), bound proteins were eluted with 100 mmol/l glycine pH 3.0 (0.5-bead volumes each) and neutralised with 1/10 volume of 1 mol/l TRIS-HCl pH 8.0. Columns were regenerated and stored in PBS/0.01 % Thiomersal (Sigma, St. Loius, Minn., USA). Aliquots of the total extract loaded (1/20) and the flow-through (1/20), as well as the wash fractions and the eluted fractions were divided into two fractions for further analysis. The first fraction was immunoprecipitated with glima-38, antibody-positive serum to identify fractions containing glima 38. The second fraction was subjected to precipitation with 10 % trichloric acid, followed by an acetone wash, to analyse the protein content of the respective fractions. Precipitates were analysed by SDS-PAGE and phosphorimaging.

Results

Identification of high-titre, glima-38 antibody-positive sera. Newly diagnosed patients with Type I diabetes ($n = 44$) were analysed for glima 38 antibodies to identify high-titre, antibody-positive sera for biochemical studies. Amongst those, ten (22.7 %) were glima-38, antibody-positive (Table 1). In the cohort of 44 patients, the prevalence of GAD65 autoantibodies (GADA) was 79.5 % and of antibodies to IA2 (IA-2A) 84.1 % (data not shown). Glima-38, antibody-positive patients were younger than antibody-negative patients (mean age ± SD; median: 14.1 ± 13.1; 10 years; vs 24.2 ± 14.5; 22 years; Wilcoxon rank test: $p = 0.049$). Patients below the age of 17 years (median of study group), were twice as frequently positive for glima 38 antibodies than patients older than 17 years (28.6 % and 13.6 %). Of the ten glima-38, antibody-positive patients, eight were positive for GADA and all ten for IA-2A (Table 1). Glima 38 antibodies were also found before clinical onset of diabetes in two ICA-positive and IAA-positive but GADA-negative and IA-2A-negative, first-degree relatives (Table 1, No. 11 and 12). One of the two glima-38, antibody-positive relatives (U122) developed Type I diabetes 2 years after the serum was collected. Glima 38 antibodies disappeared within 6 months after clinical diagnosis of Type I diabetes in one of the four subjects from whom consecutive serum samples were available (U38). The other three remained glima-38, antibody-positive over a period of 2 weeks – 0.6 years (U9, U47, U76). Sera from patients 5, 9 and 11 and plasma from patient 9 (Table 1), who were strongly positive for glima 38 autoantibodies, were used for biochemical analysis in this study.

Glima 38 is not recognised by BB-rat sera. Glima 38 antibodies have only been described in human Type I diabetes [4]. One group [14, 16] reported that sera from *BB*-rats immunoprecipitate a 38 000M_r protein in membrane extracts of isolated islet cells from *BB*-rats. This 38 000 M_r antigen is a well-defined narrow band on SDS-gels, suggesting that it is not glycated [14, 16]. We tested five *BB*-rat sera that were antibody positive and five that were antibody negative to 38 000 M_r antigens for reactivity with glima 38 in parallel with two glima-38, antibody-positive and two negative control sera. None of the *BB*-rat sera recognised glima 38 (results not shown). Thus, it appears that glima 38 is distinct from the protein described previously [14, 16].

The N-glycan of glima 38 contains terminal sialic acids and inhibition of glycation shows a core protein of M_r 22 000. Enzymatic deglycation of glima 38 in vitro, using PNGase F results in a protein of M_r 22 000 [4]. To find whether PNGase F removes all *N*-linked carbohydrates in the protein, tunicamycin, which blocks *N*-glycation in vivo, was included in biosynthetic labelling experiments of glima 38. In the presence of tunicamycin, glima 38 is synthesised as a protein of M_r 22 000 (Fig. 1A), consistent with results using PNG-

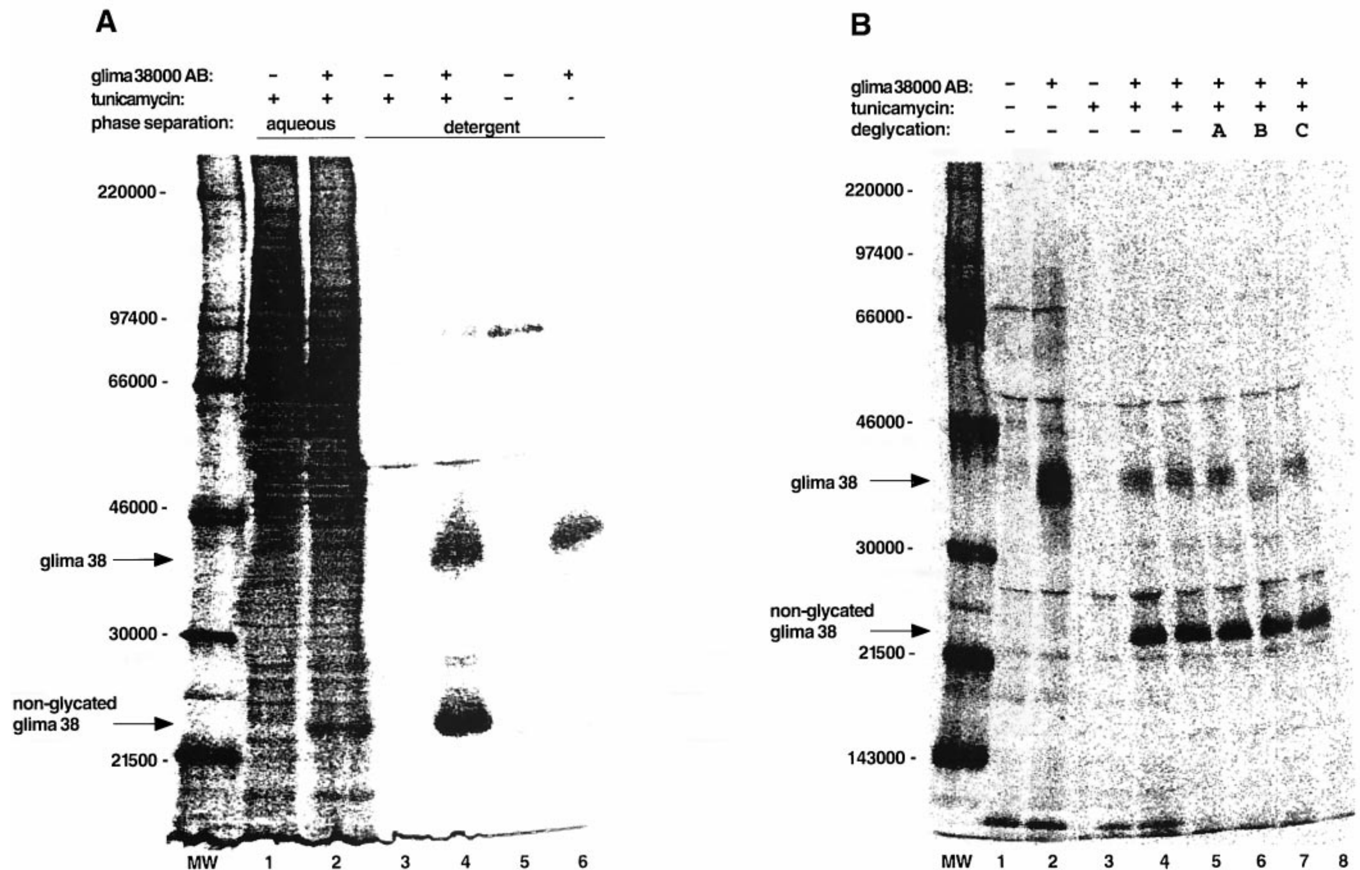

Fig. 1 A, B. Characterisation of the glycation of glima 38. SDS-PAGE analysis of immunoprecipitates of non-glycated and deglycated glima 38. **A** Inhibition of *N*-glycation of glima 38 in vivo. Immunoprecipitates of aqueous or detergent phase extracts of βTC3 cells radiolabelled in the presence (lanes 1–4) or absence (lanes 5–6) of tunicamycin. Immunoprecipitation was carried out using normal human serum (lanes 1, 3, and 5) or a glima 38 antibody positive serum (lanes 2, 4, and 6). Inhibition of *N*-glycation in vivo shows a M_r 22 000 amphiphilic core protein of glima 38. **B** Enzymatic deglycation of glima 38 in vitro. Autoradiography by phosphorimaging of an SDS-PAGE analysis of immunoprecipitates of detergent phase extracts of βTC3 cells radiolabelled in the presence or absence of tunicamycin. Samples were subjected to incubations with PNGase F (A, lane 6), neuraminidase (B, lane 7), *O*-glycosidase (C, lane 8) or IP buffer (lane 5) at 37 °C for 18 h. Immunoprecipitation was with normal human serum (lanes 1 and 3) or a glima 38 antibody-positive serum (lanes 2 and 4–8). Mature glima 38 contains terminal sialic acids, whereas the M_r 22 000 form appears to represent the non-glycated core protein of glima 38

ase. Pulse-chase experiments showed that glima 38 is fully glycated in vivo within 1 h (results not shown). To further evaluate whether the M_r 22 000 component derived from glima 38 is fully deglycated, immunoprecipitates of the M_r 22 000 protein (obtained by tunicamycin treatment) were incubated with PNGase F, neuraminidase, or *O*-glycosidase, respectively. None of these enzymes mediated a shift of the mobility of the M_r 22 000 protein on SDS-gels (Fig. 1B), suggesting that the M_r 22 000 protein is the non-glycated core of glima 38. Neuraminidase treatment resulted, however, in a shift of the mobility of the glycated form of glima 38, indicating the presence of terminal sialic acids in the carbohydrate moiety (Fig. 1B, lane 7). The *O*-glycosidase did not affect the mobility of either the M_r 38 000 or M_r 22 000 forms of glima 38 on SDS-PAGE (Fig. 1B, lane 8), suggesting that the protein does not contain *O*-linked carbohydrate moieties. In the absence of a positive control, the evidence is, however, only suggestive. In conclusion, more than about 40 % of the relative molecular weight of glima 38 is contributed by carbohydrates. Since one oligosaccharide chain of glycoproteins equals on average M_r 300–3000, glima 38 is likely to contain a minimum of five carbohydrate chains and is thus heavily glycated.

Evidence for different glycation patterns of glima 38 in pancreatic beta cells and neuronal cells. Glima 38 is expressed in pancreatic as well as in neuronal cell lines [4]. Immunoprecipitation and SDS-PAGE analysis of glima 38 from βTC3 (pancreatic) and GT1.7 (neuronal) cell lines shows that the neuronal protein has a lower mobility than the beta-cell protein on SDS-gels (Fig. 2A; lanes 3,4). Tunicamycin treatment during radiolabelling in vivo results, however, in a core protein of identical mobility from both cell types

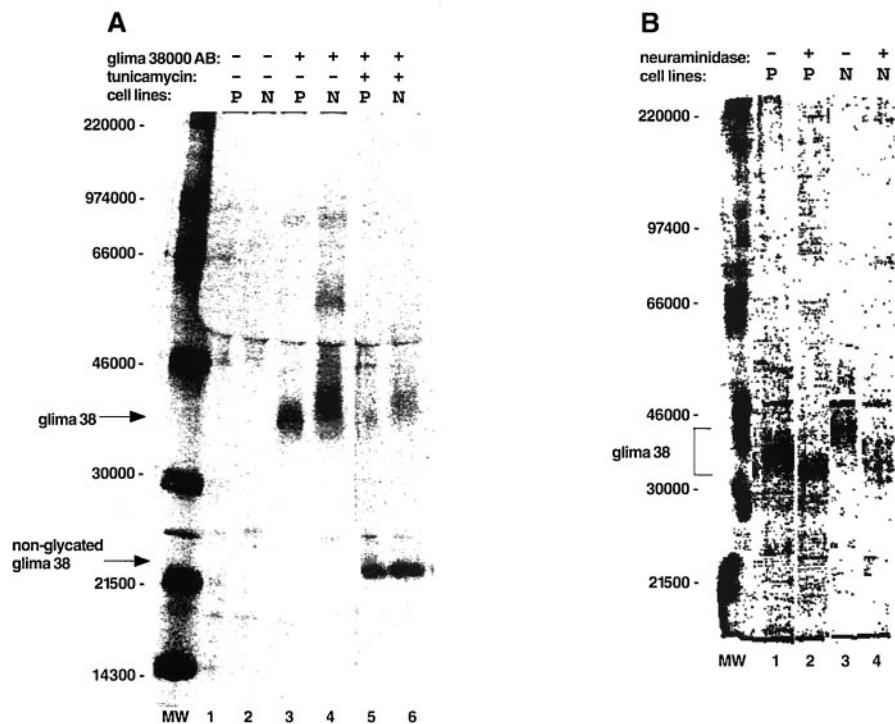

Fig. 2 A, B. Analysis of glima 38 in islet and neuronal cell lines. SDS-PAGE analyses of immunoprecipitates of detergent-phase extracts from either pancreatic βTC3 cells (P) or neuronal GT1.7 cells (N) **A** Islet cell and neuronal glima 38 share a similar protein core but differ in *N*-linked carbohydrate moieties. Cells were radiolabelled in the presence or absence of tunicamycin and immunoprecipitation was done using normal human serum (lanes 1 and 2) or a glima 38 antibody positive serum (lanes 3–6). The mobility of mature, glycated glima 38 in neuronal and beta cell lines differs, whereas the non-glycated core protein has a M_r of 22 000 in both cell types. **B** Sialylation of glima 38 in islet and neuronal cell lines. Cells were incubated with or without neuroaminidase. Glima 38 in both cell types contains terminal sialic acids, but this modification does not seem to account for the differences in mobility on SDS-PAGE

(Fig. 2A; lanes 5,6). These results suggest that the core protein is identical but that the glycation patterns differ in the two cell types. The distinct mobilities do not seem to result from differences in sialylation because a neuraminidase treatment of glycated glima 38 results in a similar shift in mobility on SDS-polyacrylamide gels (Fig. 2B). Rather, the earlier steps in glycation of glima 38 could differ between beta cells and neurons.

B-cell epitopes in glima 38 are dependent on conformation but do not require the oligosaccharide moiety. Autoantibodies to glima 38 in sera from Type I diabetic patients recognise the glycated and non-glycated forms of glima 38 equally well (Fig. 1), suggesting that the carbohydrate moiety is not a part of the autoreactive B-cell epitopes. To assess whether the autoreactive epitopes in glima 38 are dependent on conformation, the five sera of highest immunoreactivity identified in this study were incubated with denatured glima 38 on western blots. Immunoprecipitates of glima 38 were subjected to denaturing SDS-PAGE followed by blotting of protein to PVDF membrane and probing with sera at serial dilutions. None of the sera recognised glima 38 on western blots suggesting that as for GAD65 and IA-2, the autoimmune epitopes are conformational rather than linear (data not shown).

Endoprotease treatment of glima 38 using Lys-C, Asp-N and Glu-C/V8 shows specific peptide fragments. To evaluate potential cleavage sites and generate peptide maps of glima 38, endoproteinase digests were done on the protein isolated by immunoprecipitation and eluted from protein A Sepharose. Because the serum used for those studies is also weakly positive for IA-2A, IA-2; transcribed and translated in vitro; was immunoprecipitated and processed in parallel to identify peptides potentially derived from IA-2. In preliminary experiments, using a variety of incubation conditions, glycated glima 38 remained intact (data not shown), suggesting that the carbohydrate modification renders the protein resistant to proteolysis (results not shown). When proteinase incubations were carried out using deglycated protein, several specific peptides were, however, generated (Fig. 3). Thus, the treatment of immunoprecipitated and deglycated protein with Lys-C resulted in specific peptide fragments of M_r 4500 and M_r 20 000 (Fig. 3A, lanes 3,4). These fragments are likely to be derived from glima 38 and not from low levels of IA-2 present

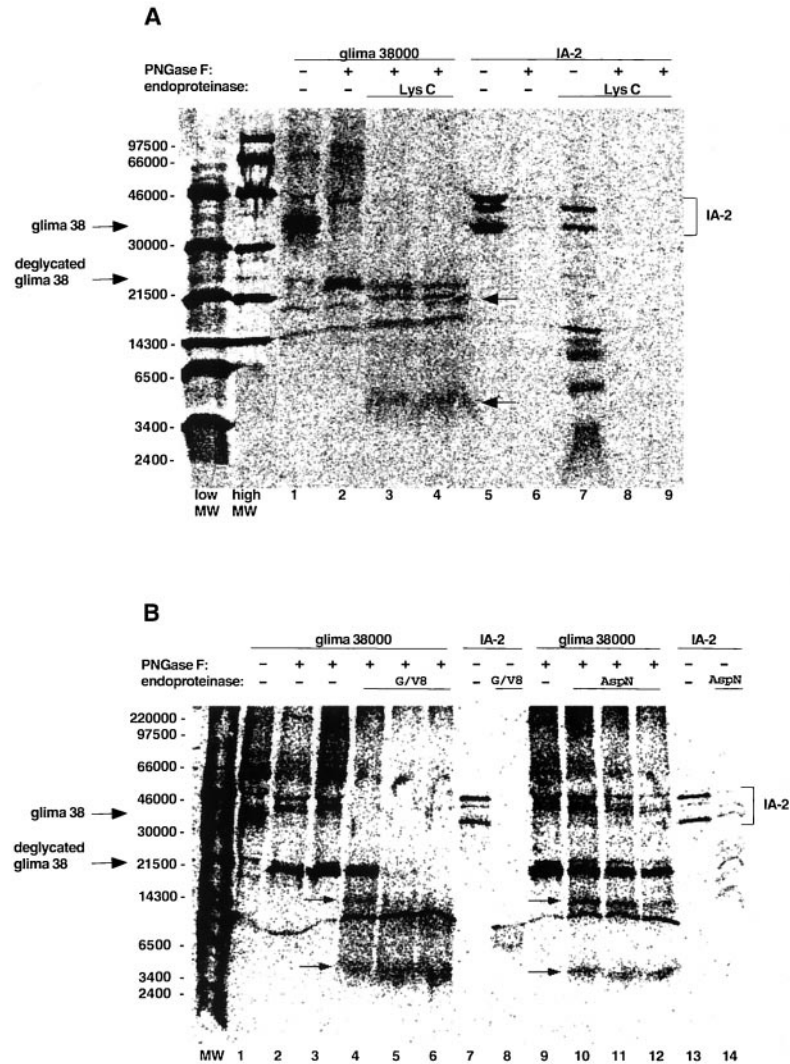

Fig. 3 A, B. Peptide mapping of glima 38. SDS-PAGE analyses of glima 38 immunoprecipitated from βTC3 cells (serum U76) and incubated with endoproteinases. **A** Lys-C digestion. Immunoprecipitates were incubated with buffer (lane 1), PNGase F (lane 2) or PNGase F followed by treatment with 0.15 U (lane 3) or 0.3 U of Lys-C (lane 4). IA-2 generated by in vitro transcription/translation was immunoprecipitated in parallel with the same serum and incubated similarly with buffer (lane 5), PNGase F (lane 6), or PNGase F followed by 0.15 U (lane 8) or 0.3 U Lys-C (lane 9). Lys-C digestion of deglycated glima 38 results in the appearance of two specific bands at M_r 4500 and 20 000 respectively (indicated by arrows), which are not detected in Lys-C digests of IA-2. **B** Glu-C/V8 and Asp-N digestion. Immunoprecipitates were treated with buffer (lane 1), PNGase F (lanes 2 and 9) or PNGase F followed by incubation with Glu-C/V8 (lanes 4–6) or Asp-N (lanes 10–12). In vitro transcribed/translated IA-2 was immunoprecipitated with the same serum and incubated (with or without a prior deglycation treatment) in parallel. Fragments that seem to be specifically derived from glima 38 are marked with an arrow. Glu-C/V8 digestion results in fragments of M_r 4000 and 12 000 respectively, which are not detected in IA-2 digests. Asp-N digestion similarly results in two fragments of M_r 4000 and 11 000 respectively, which are not detected in digests of IA-2. Amount of enzyme per 50 μl: Lanes 4, 5, 8: 1 μg; lane 6: 2 μg; lanes 10, 11, and 14: 0.04 μg; lane 12: 0.08 μg. Incubation time for lanes 3 and 4. 3 h and lanes 5, 6, 8, 11, 12, and 14, 18 h

in the immunoprecipitates for the following reasons: Firstly, IA-2; transcribed/translated in vitro and immunoprecipitated with U77, was completely degraded when subjected to a PNGase F treatment followed by incubation with either Lys-C (Fig. 3A, lanes 8,9) or buffer (Fig. 3A, lane 6). Thus in contrast to glima 38, deglycated IA-2 is completely degraded in the conditions used here. Secondly, the peptide fragments generated by Lys-C treatment of glycated IA-2 with Lys-C resulted in peptide fragments which were distinct from the M_r 4400 and M_r 20 000 fragments generated from the preparation containing glima 38 (Fig. 3A, lane 7). Similarly, digestion of glima 38 and IA-2 using either Glu-C/V8 or Asp-N (Fig. 3B) showed in both cases the appearance of peptides specific for glima 38 of approximately M_r 4500 and M_r 11 000–12 000 (Fig. 3B, lanes 4–6, 10–12). These bands were not detected after digestion of IA-2 (Fig. 3B, lanes 8,14). Thus, each of the three proteinases generates specific glima 38 peptides.

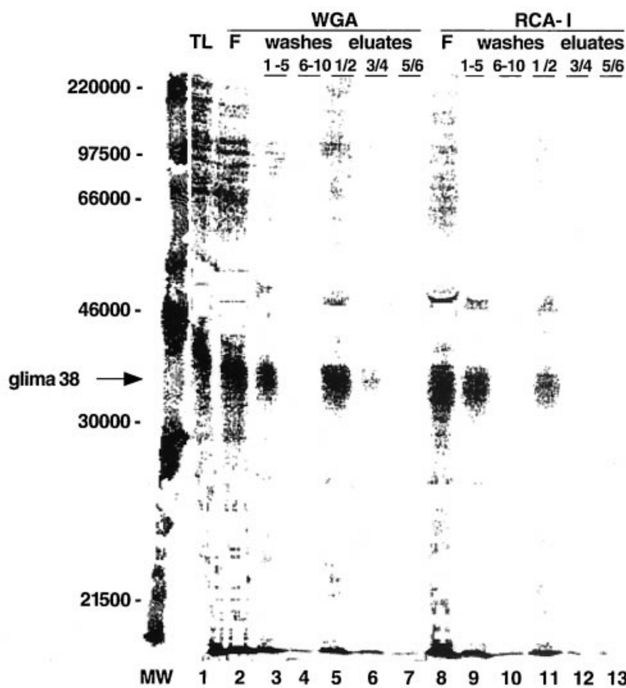

Fig. 4. Lectin affinity chromatography of glima 38. Phosphorimaging of SDS-PAGE analysis of immunoprecipitates of glima 38 from fractions obtained by affinity chromatography on colums of WGA (lanes 1–7) and RCA-1 (lanes 8–13). Lane 1 shows glima 38 in the sample applied to the columns. Glima 38 is detected in the flow through (lanes 2 and 8) and wash fractions (lanes 3, 4, 9, and 10) as well as in fractions specifically eluted from the columns (lanes 5–7 and 11–13).

Lectin binding of glima 38 shows a complex glycoprotein character. The glycated form of glima 38 binds to both castor bean lectin (RCA-I) and wheat germ agglutinin (WGA; Fig. 4A) but not to the six other lectins of different specificities tested in this study. The castor bean lectin is specific for lactose and galactose, and furthermore, binds to branched cluster oligosaccharides. Wheat germ agglutinin is specific for oligomers of (1,4)-linked *N*-acetyl-glucosamine but also reacts with terminal sialic acid residues. Although glima 38 was specifically eluted from these lectin columns using the appropriate competing carbohydrates, a fraction of glima 38 was detected in the flow-through and in the first washes, suggesting a possible heterogeneity of the carbohydrate moieties (Fig. 4A). Affinity to WGA is a characteristic of biantennary complex-type glycoproteins (*N*-linked), which also bind to RCA-I. Mucin-type glycoproteins (*O*-linked) also bind to WGA, but do not bind to RCA-I [27, 28]. Binding to lectins like ConA or LcH is a characteristic of triantennary complex-type and high mannose-type glycoproteins [27, 28]. The binding of glima 38 to WGA and RCA-I, the absence of *O*-linked carbohydrates and the lack of binding to LcH and ConA is consistent with the presence of biantennary complex-type carbohydrates in this protein.

Immunoaffinity chromatography using human polyclonal autoantibodies yields low levels of relatively pure glima 38. Different approaches were taken in attempts to purify glima 38. An immunoprecipitate of glima 38 is relatively free of contaminating ^{35}S methionine-labelled, βTC-3-cell proteins but contains several fold excess of cold IgG. Attempts to efficiently separate IgG from glima 38 by preparative SDS-PAGE were unsuccessful. We therefore immobilised IgG, purified from plasma containing glima 38 autoantibodies, on PAS to generate an immunoaffinity column. Binding and elution conditions for immunoaffinity chromatography of glima 38 were determined empirically using different buffer systems. Under alkaline conditions, elution of glima 38 was only achieved at very high pH (≥ 11.3) which was not compatible with reuse of columns. Mild acidic elution conditions enabled, however, reuse of columns and resulted in a moderate recovery of protein. Most contaminating proteins were collected in the flow-through (Fig. 5) or removed during washes from the immunoaffinity resins. Glima 38 was the predominant protein ($\sim 40\%$ of total protein) eluted under acidic conditions (Fig. 5). In contrast, glima 38 is not discernible in SDS-PAGE analyses of total lysates of βTC3 cells (results not shown). Thus, the immunoaffinity chromatography resulted in a major degree of purification of glima 38. In all experiments regardless of the conditions, the yield of glima 38 eluted from immunoaffinity columns (per mg IgG bound to resin) was, however, very low and only a small fraction of the yield from immunoprecipitates using similar quantities of IgG (Fig. 5) suggesting a low capacity of the coupled anti-glima 38 IgG. This is likely to reflect the inherent problem of using a polyclonal IgG for immunoaffinity chromatography compounded with the relatively low titre of diabetic autoantibodies. Furthermore, the coupling reaction could result in some loss of affinity. The low yield in the present experiments is consistent with results using GAD65 specific human autoantibodies for immunoaffinity resins (S. Baekkeskov unpublished results). The binding efficiency of the immunoaffinity resin was not enhanced using deglycated glima 38 (data not shown). Thus, although immunoaffinity using polyclonal human autoantibodies results in a high degree of purification of glima 38, it is unlikely to present an optimal method for purifying the protein.

Discussion

A prevalence of 19% and 14% was previously reported for antibodies to glima 38 in 86 newly diagnosed Type I diabetic patients and 44 prediabetic subjects, respectively [4]. This study found a similar prevalence (22.7%) in 44 new-onset patients. Thus, approximately every fifth patient has an ongoing im-

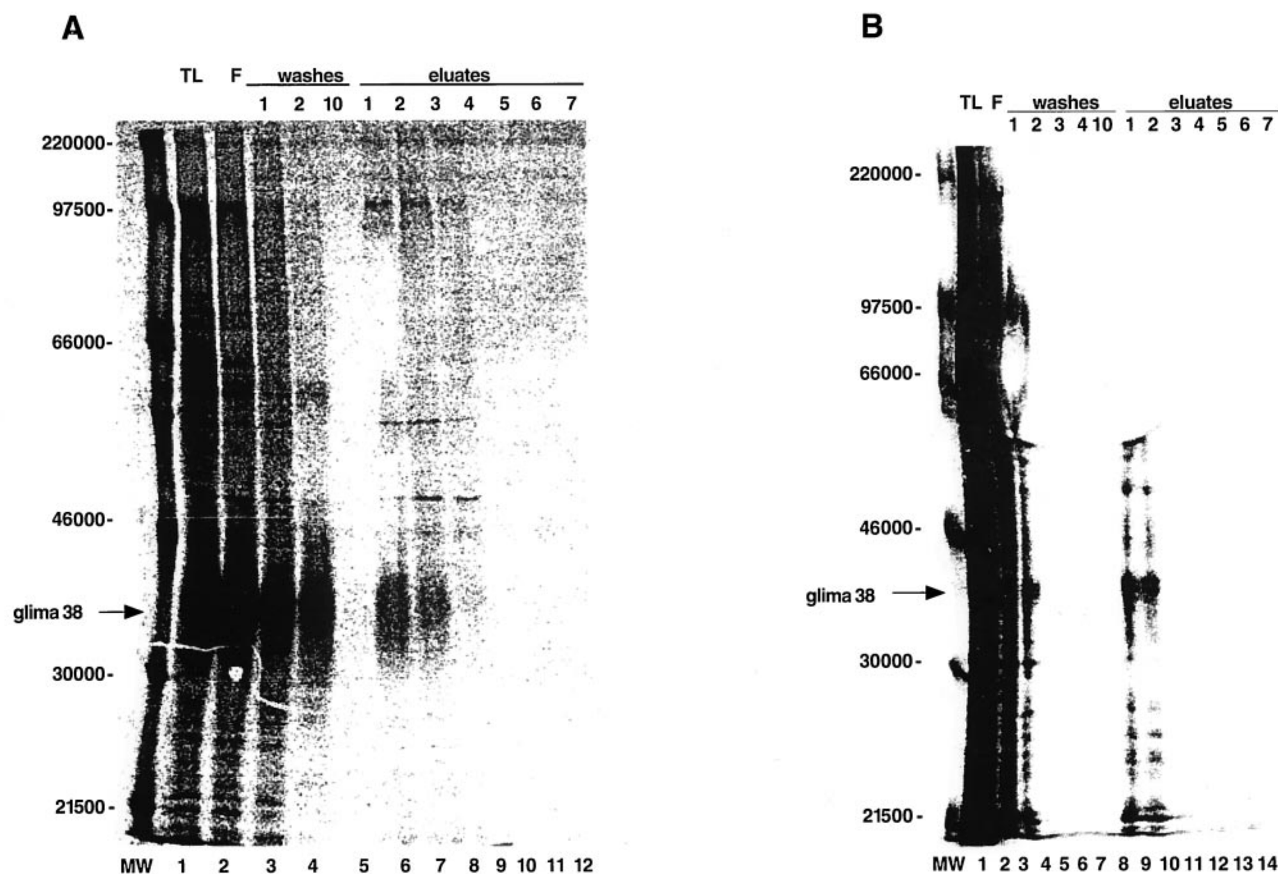

Fig. 5 A, B. Immunoaffinity chromatography of glima 38. SDS-PAGE analyses of immunoprecipitates (**A**) and TCA precipitates (**B**) of fractions obtained by immunoaffinity chromatography of a detergent phase extract of βTC3 membrane proteins using IgG purified from plasma of a glima 38 antibody positive patient. Immunoaffinity beads (0.6 ml, containing 3.4 mg bound IgG) were incubated batchwise with precleared lysate (100×10^6 cpm) and poured on a column. **A** Analysis of glima 38 in different fractions by immunoprecipitation. An aliquot (1/20, 5×10^6 cpm) of total lysate loaded (TL), 1/20 of the flow through (F), and 1/2 of the wash fractions and eluted fractions were immunoprecipitated with the U77 serum (250 µg IgG, 25 µl PAS). Because the column was overloaded to ensure maximum binding, a large fraction of glima 38 appears in the flow through (lane 2) and wash fractions (lanes 3 and 4). A small amount of glima 38 is bound to and specifically eluted from the column (lanes 6 and 7). **B** Analysis of total protein in different fractions. A duplicate of the fractions used in **A** was precipitated with TCA. Glima 38 is the major protein (~40 % of total protein) eluted in the first two fractions from the immunoaffinity column (lanes 8 and 9)

mune response to this autoantigen. In a previous study glima 38 antibodies were detected in 6 out of 44 prediabetic children up to several years before a clinical onset of Type I diabetes. Furthermore antibodies to glima 38 were detected in one of five subjects identified as ICA, GADA, and/or IA-2A positive in a general population screening of 1403 schoolchildren. The child who was positive for glima 38 antibodies developed Type I diabetes during follow-up [29]. The seven prediabetic children who were positive for glima 38 antibodies in the earlier studies were also positive for either GADA, IA-2A or both [4, 29]. The first-degree relative who was positive for glima 38 antibodies in the present study and who developed Type I diabetes during follow-up (2 years later) was, however, GADA and IA-2A negative suggesting that glima 38 autoantibodies can precede both.

Glima 38 is a *N*-linked glycoprotein with a protein core of M_r 22 000, based on two distinct analyses: (i) deglycation of mature glima 38 in vitro, using PNGase F, which cleaves *N*-linked carbohydrates at the protein core [25] and (ii) inhibition of *N*-linked glycation in vivo, using tunicamycin which blocks the coupling of *N*-linked glycans to the protein core in the endoplasmic reticulum [25]. The *O*-glycosidase failed to shift the mobility of the M_r 22 000 protein on SDS-gels suggesting that the protein does not undergo this type of glycation. We therefore conclude that deglycated glima 38 is a molecule of M_r 22 000.

Results of analysis of the carbohydrate moieties of glima 38 suggest that it is an *N*-linked glycoprotein of the biantennary complex-type [27, 28]. Notably, antibodies to glima 38 recognise both the glycated and non-glycated form of the protein, indicating that B-cell epitopes are located outside the glycated region. The biological activity of most glycoproteins does

not change when the carbohydrates are removed. In some cases saccharide moieties seem, however, to have a structural role [25, 28, 30]. For example, in chronic atrophic gastritis, binding of autoantibodies to the M_r 60 000–90 000 β-subunit of H+/K+ ATPase, which is a heavily glycated M_r 35 000 core protein with 6–7 potential *N*-glycation sites and additional *O*-glycans, requires both the specific carbohydrate and the protein moieties of the antigen. Thus, the three-dimensional protein structure is critically dependent on the presence of correctly processed glycans. For glima 38, the tertiary structure of the protein moiety itself seems to be crucial in binding of antibodies, whereas the integrity of the carbohydrate structure is not.

The IA-2 autoantigen in Type I diabetes is also *N*-glycated. It is synthesised as a M_r 105 000 core protein, which undergoes glycation resulting in a mobility corresponding to M_r 120 000 on SDS-gels [6, 8, 31]. The carbohydrate moiety in glima 38 does not, however, seem to contribute to the autoreactive epitopes in the molecule [7]. Thus, glycation of both glima 38 and IA-2 antigens is not an integral part of their autoreactive epitopes.

The different glycation of glima 38 in beta cells and neurons could be due to expression of tissue-specific glycation enzymes in the two cell types [26]. Given that glima 38 contains at least 5 oligosaccharide chains, representing more than 40 % of the final weight of the mature protein, there are many potential sites for variability in oligosaccharide synthesis. For instance, many glycoproteins from the brain contain an abundance of mannose-rich hetero-oligosaccharide chains localised predominantly in synaptosomal, myelin-enriched and microsomal fractions [32].

The peptide and glycation patterns of glima 38 described in this study are important parameters for its unequivocal identification and can, together with its membrane localisation, hydrophobicity and affinity to diabetes-specific antibodies, form the basis for its complete characterisation.

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