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A Type 1 Diabetes-related Protein from Wheat (*Triticum aestivum*)

cDNA CLONE OF A WHEAT STORAGE GLOBULIN, Glb1, LINKED TO ISLET DAMAGE*

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The development of autoimmune type 1 diabetes involves complex interactions among several genes and environmental agents. Human patients with type 1 diabetes show an unusually high frequency of wheat gluten-sensitive enteropathy; T-cell response to wheat proteins is increased in some patients, and high concentrations of wheat antibodies in blood have been reported. In both major models of spontaneous type 1 diabetes, the BioBreeding (BB) rat and non-obese diabetic mouse, at least half of the cases are diet-related. In studies of BB rats fed defined semipurified diets, wheat gluten was the most potent diabetes-inducing protein source. A major limitation in understanding how wheat or other dietary antigens affect type 1 diabetes has been the difficulty in identifying specific diabetes-related dietary proteins. To address this issue, we probed a wheat cDNA expression library with polyclonal IgG antibodies from diabetic BB rats. Three clones were identified, and the intensity of antibody binding to one of them, WP5212, was strongly associated with pancreatic islet inflammation and damage. The WP5212 putative protein has high amino acid sequence homology with a wheat storage globulin, Glb1. Serum IgG antibodies from diabetic rats and humans recognized low molecular mass (33–46 kDa) wheat proteins. Furthermore, antibodies to Glb1 protein were found in serum from diabetic patients but not in age-, sex-, and HLA-DQ-matched controls. This study raises the possibility that in some individuals, type 1 diabetes may be induced by wheat proteins. Also, it provides a first candidate wheat protein that is not only antigenic in diabetic rats and human patients but is also closely linked with the autoimmune attack in the pancreas.

Type 1 diabetes is an autoimmune disease that results when a chronic inflammatory process of unknown origin destroys most of the insulin-producing β-cells in the pancreatic islets of Langerhans. Genetic susceptibility to diabetes is inherited, and there is evidence that environmental cofactors strongly influence disease expression as follows: <50% pairwise concordance in identical twins, 3.0% annual increase in global incidence since 1960 (1), wide geographic variation, and results from numerous studies in animals showing environmental factors can modify the development of spontaneous autoimmune diabetes (2, 3). A major unresolved issue is the identification of the environmental factors that promote the development of type 1 diabetes (4). This task has proven difficult because of the multifactorial nature of the disease (4, 5), difficulty in linking past exposures to development of diabetes, lack of knowledge of the environmental antigens, and the large number of predisposing genes in individuals at risk (6).

The two most studied environmental factors are viruses and diet. Enteroviruses may be involved (7), but as yet a diabetes-inducing enterovirus has not been identified (8). Epidemiological evidence of infectious hotspots or traceable routes of infection is lacking (9), and there are conflicting data with respect to the presence of candidate viruses in the pancreas or immune cells of diabetic patients (10–12). The highest incidence of spontaneous diabetes in BB[1] rats and NOD mice occurs when they are maintained in ultraclean conditions and gnotobiotic animals still develop diabetes (13). If animals that are maintained in strict viral antibody-free conditions still develop diabetes then that leaves diet as the major environmental stimulus.

Although bovine proteins have been a central focus, a recent blinded, multicenter study demonstrated that a milk-free, wheat-based diet produced the highest diabetes frequency in diabetes-prone BioBreeding (BBdp) rats and NOD mice in three widely separate locations (14), confirming numerous reports that the highest incidence of spontaneous diabetes occurs in animals fed mixed plant-based diets in which wheat is the major component (2, 3, 15, 16). Defined diets in which wheat is the sole protein source are potent inducers of diabetes in BB rats (2, 17). In a different model of diabetes, the NOD mouse, wheat-based diets also resulted in high diabetes frequency (15, 16, 18, 19). In addition, an unusually high proportion of pa-

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[1] The abbreviations used are: BB, BioBreeding; NOD, non-obese diabetic; WP, wheat protein; ANOVA, analysis of variance; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HC, hydrolyzed casein; ORF, open reading frame; LC-MS, liquid chromatography-mass spectrometry; LSD, least significant difference; SMP-TBS, skim milk powder in Tris-buffered saline; NTP, National Toxicology Program.

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tients with type 1 diabetes (2–10%) have wheat gluten-sensitive enteropathy (celiac disease) (20), a rate that is 10–33 times that in the normal population, and ~1/3 of diabetes patients have antibodies against the celiac disease autoantigen, tissue transglutaminase (20, 21). Other reports indicate that increased peripheral blood T-cell reactivity to wheat gluten was more frequent in newly diagnosed patients (22) than in controls. These data are consistent with the involvement of dietary wheat proteins in diabetes pathogenesis.

Although considered to be a T-cell-mediated disease, studies of the prediction and pathogenesis of type 1 diabetes in humans rely heavily on serum autoantibodies as biomarkers of the destructive process. The humoral immune response to selected autoantigens correlates with histologic damage in the pancreas of newly diagnosed patients (23). Indeed, all of the major autoantigens in type 1 diabetes were identified by virtue of binding by autoantibodies from diabetic individuals. The 64-kDa autoantigen originally reported in BB rat and human islets (24, 25) was first discovered using this approach. This autoantigen was subsequently identified in patients concordant for both the neurologic disease, Stiff-man syndrome and type 1 diabetes, as glutamic acid decarboxylase, a major autoantigen in type 1 diabetes (26). Despite continued progress, the antigens that initiate and maintain the process leading to β-cell destruction remain poorly understood.

In the studies reported here, we used antibodies from rats that spontaneously develop autoimmune diabetes to identify (i) patterns of increased binding to low molecular mass wheat proteins as a function of diabetes risk and age and (ii) individual diabetes-related antigens from wheat by screening a wheat cDNA expression library. We have identified a wheat storage protein, Glb1, that is highly antigenic in diabetic BB rats, the intensity of antibody binding to this protein correlated with inflammation and damage in the pancreatic islets, and it was also recognized by IgG antibodies in serum from diabetic patients but not from controls. This report details studies that identify a first candidate diabetes-related wheat protein.

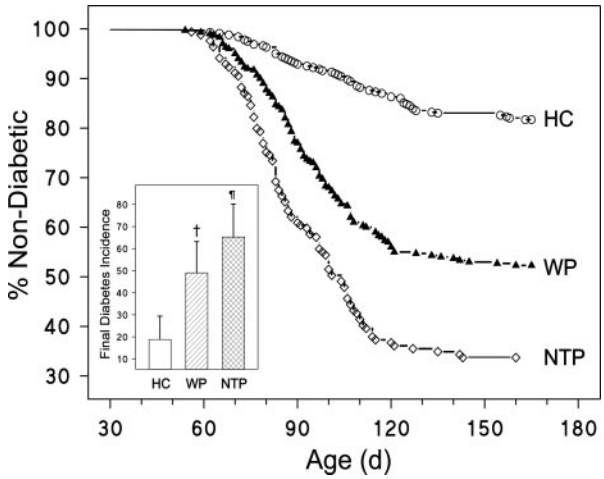

FIG. 1. **Modification of diabetes development in diabetes-prone BB rats by wheat-based diets.** Survival curves and final diabetes incidence (*inset*) in BBdp rats fed from weaning a mixed, cereal-based, mainly wheat-based, NTP-2000 (NTP) diet (31) or two semipurified, isocaloric, isonitrogenous diets in which the sole amino acid source was either HC or WP plus supplemental sulfur amino acids. Animals fed the NTP-2000 diet had the highest incidence, 65.3 ± 14.9% ($\bar{x}$ ± S.D., n = 6 experiments, total of 169 rats; ¶, denotes $p < 10^{-6}$ *versus* HC). There were more cases of diabetes in BBdp rats fed WP diets (n = 12 experiments, total of 282 rats, 50.6 ± 11.1%) than those fed a protective HC diet (n = 14 experiments, total of 322 rats, 18.8 ± 10.6%, 14 experiments; † denotes $p = 10^{-5}$).

EXPERIMENTAL PROCEDURES

Wheat cDNA Library Construction and Probing for Antigenic Proteins—Total RNA was isolated (27) from hard red spring wheat, AC Barrie, provided by Dr. V. Burrows, Eastern Cereal Oilseed Research Centre, of Agriculture and Agri-Food Canada, Ottawa, Canada. Caryopses were harvested at ~10–20 days after pollination, and total RNA was prepared and sent to Stratagene (La Jolla CA) to construct a ZAP Express® Custom cDNA library. The cDNA was inserted into the *Eco*RI/*Xho*I cloning site in the amino-terminal region of the *lacZ* gene in the ZAP Express vector (Stratagene).

XL1-Blue-MRF′ *Escherichia coli* were infected with 3.5×10^4 plaque-forming units per plate (150 × 15 mm) of phage from the wheat ZAP Express Custom cDNA library following the manufacturer's instructions (Stratagene). Protein expression was induced by the addition of 15 μl of 2 M isopropyl-1-thio-β-D-galactopyranoside per 600 μl of *E. coli*. Plaque lifts were performed, and the nitrocellulose membranes were screened following the manufacturer's instructions (Stratagene, La Jolla, CA). The primary antibody (diluted 1:200 in skim milk powder in Tris-buffered saline (SMP-TBS)) consisted of pooled sera from seven diabetic BB rats fed a wheat protein (WP) diet from weaning. The BB rat antibodies were pre-absorbed with *E. coli* phage lysate. The secondary antibody, alkaline phosphatase-conjugated AffiniPure goat anti-rat IgG, Fcγ fragment-specific antibody (Jackson ImmunoResearch, West Grove PA), was diluted 1:5000 in SMP-TBS. Antibody binding was detected using alkaline phosphatase development solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing nitro blue tetrazolium chloride (0.3 mg/ml) and 5-bromo-4-chloro-3-indolyl phos-

TABLE I
Identification and characterization of clones isolated from a wheat cDNA expression library

Clone	DNA homology, % identity/no. bp[a]	Amino acid homology, % identity/no. amino acids[a]	ORF length[b]	Putative protein length (amino acids)[c]
			bp	
WP5212	*Triticum aestivum* (wheat) storage protein (Glb 1) gene, 90%/1387	*T. aestivum* (wheat) storage protein, 80%/642	1890	629
WP12111	Clone CNW03PL453 ITEC CNW wheat powdery mildew-resistant line, *T. aestivum*, 96%/138	Unknown protein, *A. thaliana*, 63%/144	789	262
WP23112	*Arabidopsis thaliana* DNA chromosome 3, BAC clone T16L24, 96%/542 Clone TA012XXX, Brevor mature embryo ABA library (*T. aestivum*), 100%/511	Putative protein, *A. thaliana*, 62%/150	624	207
WPCON	Ascorbate peroxidase (*Hordeum vulgare*) 91%/326	Ascorbate peroxidase (*H. vulgare*), 96%/86	366	121

[a] BLASTn and BLASTx sequence homology searches were performed using the GenBank™ [2] and TIGR Wheat Gene Index[3] data bases.
[b] The open reading frame contains 95 bp from the 5′ β-galactosidase gene.
[c] Determined using Clone Manager 6® (Sci-Ed Software (2002) website address: www.scied.com/ses_cm6.htm, Scientific and Educational Software, Durham, NC).

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TABLE II
Proteins with sequence homologies to Glb1 found by BLAST (28) searches of the GenBank™ [2] and NCBI human genome data bases

Group[a]	Homologous protein[b]	Expect value[c]	Amino acid homology, % identity/no. amino acids
1	Allergen Ara h 1, clone P17 precursor (Ara h 1) Gi|1168390|sp|P43237|AL11_ARAHY	$4e^{-25}$	25%/630
	Ara h 1 commonly recognized epitope 2[b]	7.7×10^5	60%/5
	Ara h 1 immunodominant epitope 3[b]	7.7×10^5	50%/6
	Ara h 1 immunodominant epitope 4[b]	5.4×10^4	100%/4
	Ara h 1 commonly recognized epitope 8[b]	4.3×10^6	80%/5
	Ara h 1 commonly recognized epitope 12[b]	5.4×10^4	83%/6
	Ara h 1 commonly recognized epitope 13[b]	8.9×10^2	70%/10
	Ara h 1 immunodominant epitope 17[b]	7.7×10^5	80%/5
	(AB046874) allergen Gly m Bd 28K (*Glycine max*) Gi|12697782|dbj|BAB21619.1|	<0.005	21%/483
2	Root allergen protein (RAP), dandelion Gi|7388038|sp|O49065|RAP_TAROF	<688	31%/58
3	Tight junction protein, ZO-2, chicken Gi|7512238|pir||JE0366 37	<17	29%/124
	Similar to tight junction protein ZO-1 (*Homo sapiens*) Gi:17436387	<1.2	28%/142
	Tight junction protein 2 (*Zona occludens* 2); Friedreich ataxia region gene X104 (Tight junction protein ZO-2) (*H. sapiens*) Gi:4759342	<5.8	31%/133
	Similar to Tight junction protein ZO-2 (*Z. occludens* 2 protein) (tight junction protein 2) (*H. sapiens*) Gi:13639591	<5.8	31%/133

[a] Group 1 refers to homologies retrieved with and without the low complexity filter. Group 2 refers to homologies retrieved with the low complexity filter only. Group 3 refers to homologies retrieved without the low complexity filter only.
[b] Homology achieved using the sequence homology matrix PAM-30, recommended for comparing sequences less than 35 amino acids in length.
[c] Expect value is defined as follows by NCBI as a parameter that describes the number of hits one can "expect" to see just by chance when searching a data base of a particular size. The *E* value describes the random background noise that exists for matches between sequences. For example, an *E* value of 1 assigned to a hit can be interpreted as meaning that in a data base of the current size one might expect to see 1 match with a similar score simply by chance.[2]

phate (0.15 mg/ml). Positive clones were detected as dark purple plaques and cored from the agar.

The agar plugs were placed in 500 μl of SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin) containing 20 μl of chloroform and stored at 4 °C. Screening was repeated until the positive phage reached clonality. Single clone excision was performed to allow *in vivo* excision and recircularization of the cloned insert, according to the manufacturer's instructions (Stratagene). Resistance to kanamycin indicated the presence of the pBK-CMV phagemid.

Phagemid DNA was prepared for sequencing using a Plasmid Midi Kit (Qiagen, Mississauga, Ontario, Canada). The cDNA inserts were sequenced at the University of Ottawa Biotechnology Research Institute on a 373 Stretch sequencer (Applied Biosystems, Foster City, CA) using standard T3 forward and T7 reverse primers. For clone WP5212, internal primers were designed to sequence the full cDNA insert (forward, 5′-ACCACGGGTTCGTCAAGG-3′; reverse, 5′-AACACCTCCTGCACCTCC-3′). Nucleotide and translated BLAST (28) searches of the GenBank™ [2] and TIGR Wheat Gene Index[3] data bases were performed for each sequence.

Human Subjects—Blood samples for serum were obtained from Finnish children newly diagnosed with type 1 diabetes but not yet treated with insulin (n = 23; mean age 9.8 ± 3.4 years) and non-diabetic control children (n = 37; mean age 9.9 ± 3.5 years), matched for age, sex, and HLA-DQ major histocompatibility complex class II haplotype. Permission for blood sampling and ethics approval were obtained from the local ethics committee at the University of Turku.

Animals—Male and female diabetes-prone BioBreeding (BBdp) and control BB rats (BBc) were obtained from the Animal Resources Division of Health Canada (Ottawa). The animals are maintained in laminar flow protected cages under specific pathogen-free conditions. The mean incidence of diabetes in BBdp rats from this colony fed a standard cereal-based diet (31) has remained constant over the past 5 years at 65.3 ± 14.9% (mean ± S.D.). This colony is directly descended from the original diabetic rats discovered at BioBreeding laboratories near Ottawa in 1974 and transferred to Health Canada in 1977. The colony is not completely inbred but has remained a closed colony for the past 25 years, and recent genotyping for selected markers indicates the animals are ~80% identical at the DNA level. These animals carry the same mutation at the *Iddm1/lyp* locus as BB/W rats that is attributable to a frameshift deletion in a novel member of the immune-associated nucleotide-related gene family, Ian5 (32). BBc rats are derived from an early subline of animals from the original BB rat colony that does not spontaneously develop diabetes. Tests in sentinel animals indicate the colony is antibody-free with respect to Sendai virus, pneumonia virus of mice, rat corona virus/sialodacryoadenitis virus, Kilham rat virus, Toolan's H-1 virus, reovirus type 3, and *Mycoplasma pulmonis*. Animals were weaned at 23 days of age, caged in banks of 30 wire-bottom cages, and given free access to food and water. The principles of laboratory animal care as described by the Canadian Council on Animal Care were followed.

Animals were tested twice weekly for glucose in urine using Testape (Lily, Toronto, Ontario, Canada) after 60 days of age. Those with a value greater than 2+ were fasted overnight, and blood glucose in tail blood was measured the next morning using a glucometer. Diabetes was diagnosed when fasting blood glucose was >11.1 mmol/liter. Diabetic animals were killed within 24 h of diagnosis by exsanguination while under anesthesia with 3% halothane in oxygen.

Insulitis Scores—All histological analyses were performed on coded samples. Hematoxylin and eosin-stained sections of pancreas fixed in Bouin's solution were evaluated at ×100 magnification and confirmed at ×200 magnification using an Axiolab microscope (Zeiss, Mississauga, Ontario, Canada). Subjective overall rating of pancreatic islet inflammation (insulitis (33)) was performed using the following scale: 0, normal islet appearance; 1, infiltration in islet periphery only; 2, infiltration concentrated in islet periphery with infiltration in the islet core; 3, infiltration concentrated in one-third of the islet core; 4, infiltration concentrated in up to one-half of the islet core; 5, end stage islets with widespread β-cell destruction and/or core filled with infiltrating mono-

[2] NCBI (2002) website address: www.ncbi.nlm. nih. gov/BLAST/, National Center for Bio/Technology Information, National Library of Medicine, Bethesda, MD.
[3] TIGRWheat data base (2001) website address: ww.tigr.org/tdb/tagi/, Institute for Genomic Research, Rockville, MD.

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nuclear cells. The mean of 10 islets per animal was used for an overall insulitis score. Inflammation of the islets was also measured as the percent of infiltrated islets.

Diets—The NTP-2000 diet (Zeigler Bros., Gardners, PA) is an open formula (the percentage composition is known) and nonpurified diet for rodents developed by the United States National Toxicology Program of the NIEHS of the National Institutes of Health. NTP-2000 does not contain any milk protein. This is a mainly plant-based (milk-free) diet with wheat as the major component (37%), followed by corn, soybean meal, alfalfa meal, oat hulls, fish meal, and cellulose. The diet contains ~14.6% protein, 8.2% fat, 9.9% crude fiber, 52% carbohydrate, 10.7% moisture; the remainder is native and added micronutrients. The NTP-2000 diet used in these studies was irradiated and contained low levels of chemical and microbial contaminants (31). WP semipurified diets were made up of 22.5% wheat gluten (ICN Biochemicals, Cleveland, OH), 50.2% corn starch, 12.0% sucrose, 5.0% corn oil, 5.0% fiber (Solka-Floc), 3.5% AIN-76 (or AIN-93G) mineral mix (ICN), 1.0% AIN-76A (or AIN-93G), vitamin mix (ICN), supplemented with 0.2% choline bitartrate, 0.02% DL-methionine, 0.5% L-lysine, and 0.08% L-threonine to compensate for low sulfur amino acids in wheat proteins. Hydrolyzed casein (HC) diets contained 51.0% corn starch, 12.0% sucrose, 20.0% casein hydrolysate (pancreas S enzymatic hydrolysate, Redstar Bioproducts, Mississauga, Ontario, Canada), 7.0% soybean oil, 5.0% fiber, 3.5% AIN-76 (or AIN-93G) mineral mix, 1.0% AIN-76A (or AIN-93G) vitamin mix, 0.2% choline bitartrate, and 0.3% L-cystine. Both semipurified diets were isocaloric and isonitrogenous.

Probing Wheat Clones for Antibody Reactivity Using Serum from Individual Rats Fed WP-based Diets—Serum (diluted 1:200 in SMP-TBS) from individual diabetic (n = 7), asymptomatic (no clinical symptoms of diabetes by 150 d; n = 10) BBdp, and BBc (n = 9) rats was used to screen the wheat clones in the same manner as for the library screening. Densitometric analysis of regions of interest on nitrocellulose blots of wheat clones was performed using a Kodak Digital Science™ image station 440CF. The mean intensity/pixel for each region of interest was tabulated. A clone was randomly chosen from the library to represent background antibody binding. This clone, WPCON, had an ORF 366 bp long and an expected expression product size of 121 amino acids (Table I). WPCON shared 91% identity across 326 nucleotides with barley ascorbate peroxidase mRNA (*Hordeum vulgare*, GenBank™ accession number AF411228.1) and shared 96% identity across 86 amino acids with the ascorbate peroxidase protein (*H. vulgare*, GenBank™ accession number AAL08496.1).

One-dimensional Western Immunoblotting of Wheat Proteins—Proteins were extracted from wheat gluten powder (ICN) using lysis buffer as described previously (34). Samples were electrophoresed in 10% SDS-PAGE gels (35), transferred to nitrocellulose, and blocked with 5% (w/v) SMP-TBS, pH 7.5. Blots were incubated with sera diluted in SMP-TBS, 1:600. Samples were from rats at different risk of diabetes and fed WP diet as follows: control BBc (n = 10), asymptomatic BBdp (no clinical symptoms of diabetes by 120 days, n = 7), and pre-diabetic BBdp animals (developed overt diabetes before 120 days, n = 7, or animals with overt diabetes, BBd). Sera from individual patient (n = 23) and non-diabetic HLA-DQ-matched control children (n = 37) were diluted 1:50. Following 5 times 5-min washes with TBS containing 1% (v/v) Tween 20, the membrane was exposed to horseradish peroxidase-conjugated goat anti-rat IgG (Fcγ-fragment specific; Cedarlane Laboratories, Hornby, Ontario, Canada) or rabbit anti-human total IgG antibody (Dako), diluted 1:2000 with SMP-TBS. Bands were visualized using ECL according to the manufacturer's instructions (ECL-Western blotting detection, Amersham Biosciences) and quantified by densitometry. Digital images of the Western blot films were acquired using the Kodak Digital Science™ image station 440CF (Rochester, NY) and analyzed using the Kodak Digital Science™ one-dimensional image software (New Haven, CT).

Two-dimensional Western Immunoblotting of Wheat Proteins—150 μg of wheat gluten proteins in lysis buffer were added to the IEF buffer (4% CHAPS, 7 M urea, 2 M thiourea, 40 mM Trizma base, 2 mM tributylphosphine, and 0.4% Bio-lyte 3/10 (Bio-Rad)) and applied to rehydrated Ready Strips (Bio-Rad) with an immobilized linear pH gradient from pH 3 to 10. Wheat proteins were focused at 21 °C for a total of 100,000 V-h on the Protean IEF cell (Bio-Rad), reduced with 20 mg/ml of dithiothreitol, and alkylated with 25 mg/ml iodoacetamide. Proteins were separated in the second dimension in 10% SDS-PAGE gels by electrophoresis at 30 mA for 15 min and 60 mA for 2 h, transferred to nitrocellulose membrane, and blocked using SMP-TBS overnight at 4 °C. Serum was pooled from the 23 patients and 37 controls, diluted 1:500 in SMP-TBS buffer, and used to probe two-dimensional Western blots (1 h at 4 °C). The secondary antibody, rabbit anti-human total IgG conjugated with horseradish peroxidase (Dako), was diluted to 1:2000 with SMP-TBS buffer and incubated with the membranes for 30 min at 4 °C. Antibody binding was visualized using ECL as recommended by the manufacturer (Amersham Biosciences) and analyzed using two-

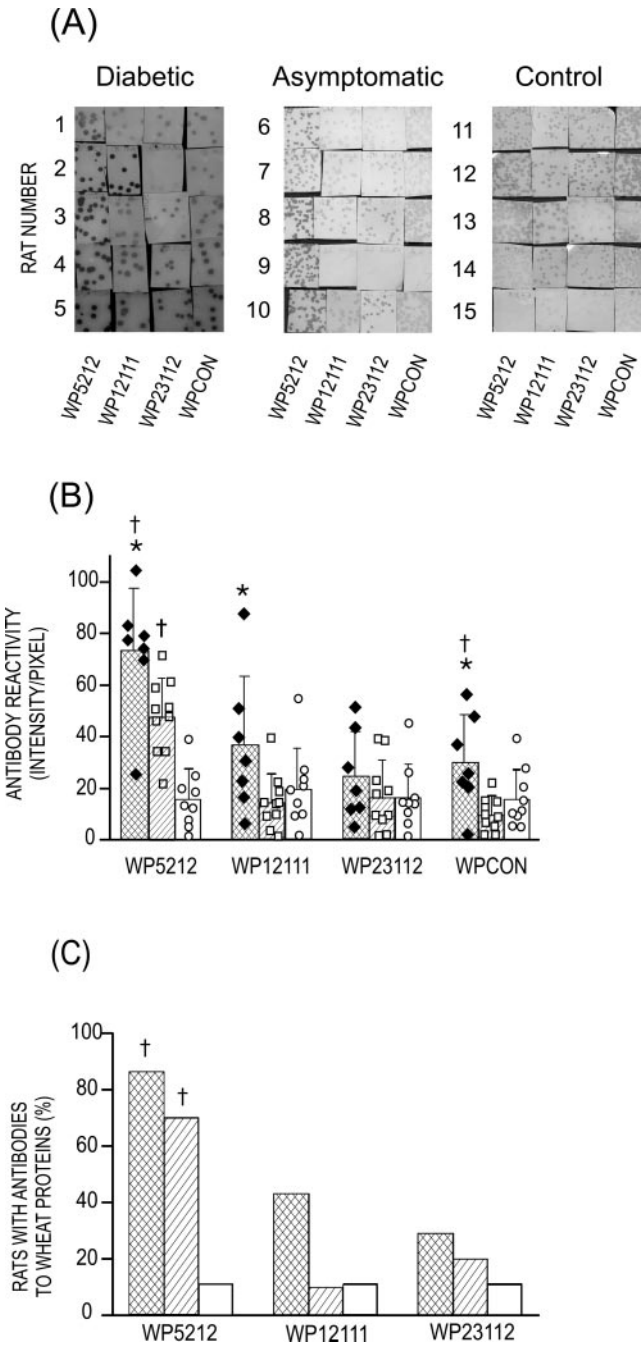

FIG. 2. **Examples of plaque lifts of clones screened with serum from diabetic, asymptomatic, or control rats (*panel A*), antibody reactivity to four clones (*panel B*), and frequency of antibody reactivity to the wheat proteins (*panel C*).** *Panel A*, plaque lifts of clones WP5212, WP12111, WP23112, and WPCON screened with serum from five representative diabetic, asymptomatic, or control rats. *Panel B*, mean antibody reactivities (intensity/pixel) ± S.D. to the recombinant wheat proteins screened with diabetic (n = 7, *cross-hatched bars*), asymptomatic (n = 10, *hatched bars*), or control (n = 9, *open bars*) BB rats are shown. Individual values for diabetic (*diamonds*), asymptomatic (*squares*), or control (*circles*) rats are shown. *Panel C*, the frequency of diabetic (*cross-hatched bars*), asymptomatic (*hatched bars*), and control (*open bars*) BB rats with positive antibody reactivity to the wheat proteins is shown. A positive antibody level was defined as an antibody reactivity greater than the mean intensity of WPCON screened with control rat serum plus two S.D. (ANOVA/LSD; † indicates significant difference *versus* control rats, $p \leq 0.02$; * indicates significant *versus* asymptomatic rats, $p \leq 0.02$).

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TABLE III
Number of islets and inflammation in the pancreas of rats whose serum was used to screen wheat clones

BB rat	Total no. islets	% islets infiltrated	Mean insulitis score
Diabetic (n = 7)	39 ± 19[a–c]	63.4 ± 20.6[b,c]	2.9 ± 1.0[b,c]
Asymptomatic[d] (n = 10)	60 ± 29	41.4 ± 21.5[c]	1.3 ± 0.7[c]
Control (n = 9)	72 ± 13	5.0 ± 5.3	0.2 ± 0.1

[a] All values are mean ± S.D.
[b] Significantly different from asymptomatic (one-way ANOVA/LSD, $p \leq 0.03$).
[c] Significantly different from control (one-way ANOVA/LSD, $p \leq 0.002$).
[d] Asymptomatic refers to animals without clinical symptoms of diabetes by age 150 days.

dimensional analysis software (PDQuest, Bio-Rad).

Mass Spectrometry Analysis—Two-dimensional gels of wheat gluten proteins were stained with a non-fixing silver stain (36). Excised gel plugs were digested overnight at 37 °C with 200 ng of modified sequencing grade trypsin (Promega) in the ProGest™ automatic digester (Genomic Solutions, Ann Arbor, MI) as described (36). Rapid capillary LC-MS/MS was performed using a Waters CapLC liquid chromatograph (Waters, Milford, MA) coupled to a Q-TOF2 mass spectrometer (Micromass, Manchester, UK) with an electrospray ionization interface. The digested extracts were redissolved in 5% (v/v) acetonitrile, 0.5% acetic acid, and 10 μl was loaded onto a 0.3 × 5 mm C_{18} micro precolumn cartridge (Dionex/LC-Packings) for each analysis. Rapid peptide elution was achieved using a linear gradient of 5–60% acetonitrile, 0.2% formic acid in 6 min (flow rate of 1 μl/min). The mass spectrometer was operated in data-dependent acquisition mode.

Statistical Analysis—Comparisons between sample populations were made using one-way ANOVA and Scheffe's or LSD post hoc tests (Statistica version 4.5, StatSoft Inc., 1993, Tulsa, OK). Fisher's Exact test (two-tailed) was used to compare the frequency of individuals with antibody reactivity to wheat proteins. Pearson Product-Moment correlation was used to determine r and p values. Survival analysis using the log Rank test was used to compare the effect of different diets on diabetes incidence (Statistica).

RESULTS

Wheat Protein Diets Can Modulate Diabetes Outcome—Animals fed a non-purified, defined, mainly wheat-based (31), NTP-2000 diet showed the highest incidence of diabetes (n = 6 experiments, total of 169 rats, 65.3 ± 14.9%, Fig. 1). When comparing only defined, isocaloric, and isonitrogenous semi-purified diets with amino acids from wheat gluten or hydrolyzed casein, there were more cases of diabetes in BBdp rats fed WP diets (n = 12 experiments, total of 282 rats, 50.6 ± 11.1%) compared with BBdp rats fed a protective HC diet (n = 14 experiments, total of 322 rats, 18.8 ± 10.6% Fig. 1; ANOVA/LSD, $p < 1 \times 10^{-5}$).

Three Immunogenic Wheat Clones Isolated from a Wheat cDNA Expression Library—A wheat cDNA expression library consisting of over one million recombinant phage was generated. The primary screening of the library, using pooled diabetic BB rat serum (n = 7), yielded 48 positive clones. Eight of these were found to be true positives and were repeatedly screened until they reached clonality. The eight clones could be categorized into three groups based on cDNA insert size (2.1, 1.1, and 0.8 kb). Sequencing the cDNA inserts confirmed the presence of three distinct sets of positive clones (Table I). Representative clones, WP5212, WP12111, and WP23112, from each distinct set were used for all further analyses.

Nucleotide and translated BLAST searches of GenBank™[2] and TIGR Wheat Gene Index[3] data bases were performed (Table I). Clone WP5212 contained a 1890-bp open reading frame (ORF), including 95 bp of the *lacZ* gene. It shared 90% identity across 1387 nucleotides with the *Triticum aestivum* wheat storage protein (Glb1) gene (GenBank™ accession number M81719.1). The expected translated amino acid sequence was 629 amino acids in length and shared 80% identity across 642 amino acids with the *T. aestivum* wheat storage protein (GenBank™ accession number AAA34269.1), Glb1.

WP5212 also shared sequence homologies with the peanut allergen Ara h 1 (GenBank™ accession number P43237; 25% identity across 630 amino acids; Table II), which is associated with food-induced type 1 hypersensitivity. The antibody-binding epitopes have been mapped for Ara h 1, and WP5212 shares homology with three of four immunodominant epitopes, as well as four of five other commonly recognized epitopes (37). WP5212 also had sequence homology with two other plant allergens, a soybean protein (*Glycine max*, GenBank™ accession number BAB21619) and a dandelion root protein (*Taraxacum officinale*, GenBank™ accession number RAP_TAROF). A BLAST search of the human genome and NCBI data bases retrieved sequence homologies to tight junction protein 2 (*Homo sapiens*, accession number 4759342, and *Gallus gallus*, accession number 7512238), similar to tight junction protein ZO-1 (*H. sapiens*, accession number 17436387) and similar to tight junction protein ZO-2 (*H. sapiens*, accession number 13639591).

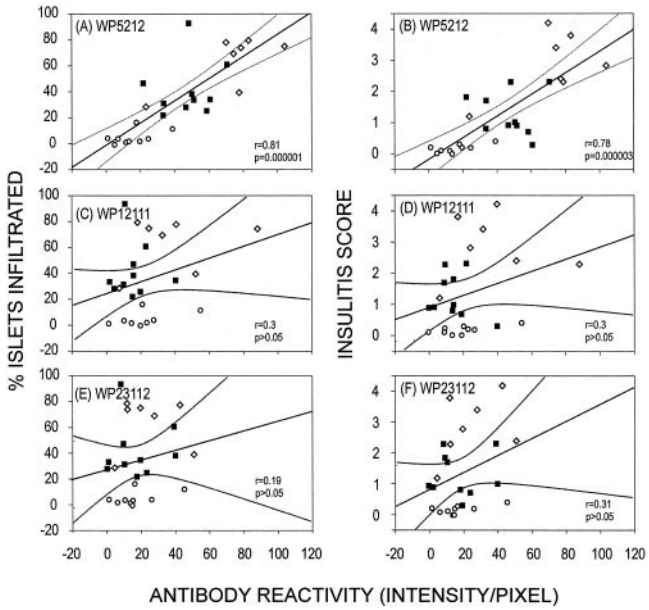

FIG. 3. **Antibody reactivity to the Glb1 clone is strongly associated with pancreatic inflammation or insulitis.** The correlations between the percent of islets infiltrated (*left column*) or insulitis score (*right column*) and antibody reactivity (mean intensity/pixel) to three recombinant wheat proteins in diabetic (*diamonds*), asymptomatic (*squares*), or control (*circles*) rats are shown. The Pearson Product-Moment correlation r and p values are indicated.

The ORF for cDNA clone WP12111 was 789 bp coding for a putative product of 262 amino acids (Table I). The nucleotide sequence shared 96% identity across 138 nucleotides with clone CNW03PL453 ITEC CNW from a wheat powdery mildew-resistant line library (accession number BE401554) and 63% identity across 144 amino acids with an unknown *Arabidopsis thaliana* protein (accession number AAK25945).

Clone WP23112 had an ORF of 624 bp coding for a putative product of 207 amino acids (Table I). WP23112 shared 96% identity across 542 nucleotides with the BAC clone T16L24 from *A. thaliana* DNA chromosome 3 (accession number 6899943) and 62% identity across 150 amino acids with the

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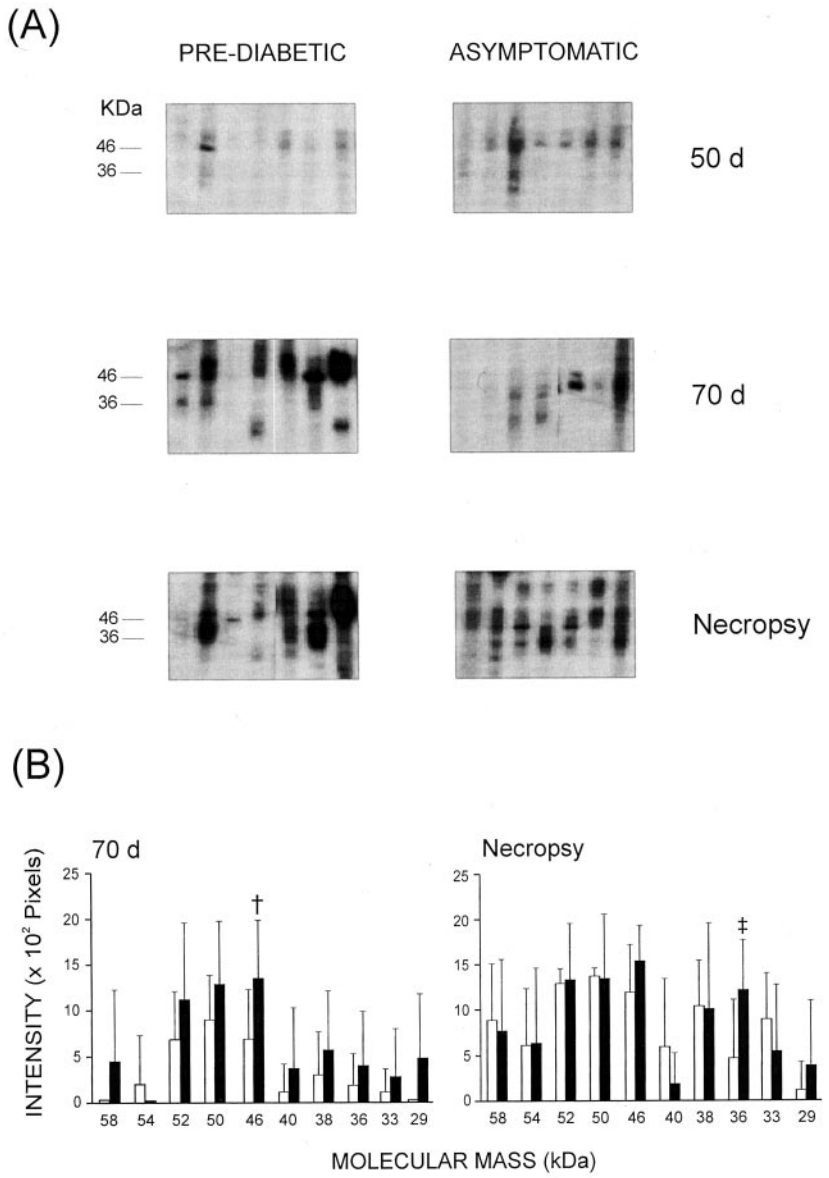

FIG. 4. **One-dimensional Western analysis of wheat proteins probed with serum collected prospectively from BB rats at different risk of developing diabetes.** One-dimensional Western blots of wheat proteins probed with serum from prediabetic or asymptomatic BB rats at 50 and 70 days and at necropsy are shown in *A*. The mean intensity ± S.D. of each wheat protein band is shown for the prediabetic period (70 days) or at necropsy for asymptomatic (*open bars*) or diabetic (*filled bars*) in *B*; ANOVA/LSD; †, $p = 0.02$; ‡, $p = 0.006$.

gene product, a putative *A. thaliana* protein (accession number CAB75463.1). Clone WP23112 also shared 100% identity across 511 nucleotides with a clone from a Brevor mature wheat embryo ABA library (accession number WHE0606).

Diabetic Rats have Increased Frequency and Intensity of Antibody Reactivity to Glb1—To determine whether antibody reactivity to WP5212, WP12111, and WP23112 was related to diabetes risk, the clones were screened with serum antibodies from individual diabetic ($n = 7$), asymptomatic ($n = 10$), and control ($n = 9$) BB rats (Fig. 2, *panels A* and *B*). Antibody reactivity was measured by densitometry and is reported as intensity/pixel. Antibody reactivity to WP5212 in diabetic rats was significantly higher than in asymptomatic ($p = 0.005$) and control ($p = 10^{-6}$) rats. Asymptomatic BBdp rats also had increased antibody reactivity to WP5212 compared with control rats ($p = 0.0004$). Diabetic rats had higher antibody reactivity to WP12111 than asymptomatic rats ($p = 0.02$). Antibody reactivity to WP23112 did not differ among the rat groups. Diabetic rats had increased antibody reactivity to WPCON compared with asymptomatic and control rats. Antibody reactivity in serum from BB control rats was not different among any of the proteins analyzed, suggesting that this level represented nonspecific antibody reactivity.

The frequency of rats with antibodies to wheat proteins was determined (Fig. 2, *panel C*). A positive antibody level was defined as an antibody reactivity value greater than the mean intensity plus 2 S.D. for WPCON screened with control serum. More diabetic ($p = 0.009$) and asymptomatic ($p = 0.02$) rats had antibodies to WP5212 than control rats, but there was no difference in frequency of antibody reactivity to WP5212 between diabetic and asymptomatic rats. There was no difference in frequency of antibody reactivity to WP12111 and WP23112 among the rat groups.

Antibody Reactivity to a Glb1 Protein Correlates with Islet Inflammation and Damage—To determine whether antibody reactivity to the cloned wheat proteins correlated with damage to the target tissue, the proportion of islets infiltrated with mononuclear cells was calculated, as well as the mean insulitis score. A relationship with diabetogenesis was considered to occur when both percent infiltration (degree of inflammation) and mean insulitis score showed a significant correlation with antibody intensity on the dot blots. Diabetic rats had significantly fewer islets than both asymptomatic and control rats (Table III). There was no difference in total islet number between asymptomatic and control rats. Diabetic rats had a higher percent of infiltrated islets and mean insulitis score

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compared with both asymptomatic ($p = 0.02$ and $p = 0.0001$) and control ($p = 10^{-6}$ and $p < 10^{-7}$) rats. In asymptomatic rats, the percent of infiltrated islets was higher, as was the mean insulitis score compared with control rats ($p = 0.0001$ and $p = 0.002$). A positive correlation was observed between antibody intensity to WP5212 and percent of infiltrated islets (Fig. 3, *panel A*, $r = 0.81$, $p = 10^{-6}$) and mean insulitis score (Fig. 3, *panel B*, $r = 0.78$, $p = 3 \times 10^{-6}$). There was no correlation between antibody reactivity to WP12111 or WP23112 and percent of islets infiltrated and mean insulitis score (Fig. 3, *panels C–F*).

Increased Humoral Immune Reactivity to Low Molecular Mass Wheat Proteins in Pre-diabetic Rats—To examine whether differences in antibody binding to wheat proteins were associated with the development of disease, Western blots of wheat gluten proteins were probed with serum obtained prospectively at 50 and 70 days from BB rats at different risk of developing diabetes. Western blots of wheat proteins showed antibody reactivity increased with age in BBdp rats (Fig. 4, *panel A*). At day 50 the level of antibodies in asymptomatic and pre-diabetic rats was similar. Compared with animals that remained asymptomatic, higher signal intensity was detected for wheat proteins around 46 kDa ($p = 0.02$, Fig. 4, *panel B*) in prediabetic animals at approximately day 70. At necropsy, animals with overt diabetes had stronger reactivity to 36-kDa wheat proteins compared with asymptomatic rats ($p = 0.006$). Blots probed with BBc rat serum at 1:600 showed low antibody binding to wheat proteins (data not shown). The frequency of rats reacting to these wheat proteins did not differ when comparing BBc, BBdp, or overt diabetic animals.

One-dimensional and Two-dimensional Western Blots Show Increased IgG Binding to Wheat Proteins in Patient Serum; Glb1 Protein Is Bound by Antibodies from Patients but Not Controls—One-dimensional Western blots were used to investigate antibody binding to wheat proteins (Fig. 5, *panel A*). Signal intensity for the 33-kDa proteins was higher in patients than in controls in 19 of 23 case control comparisons (83%), whereas it was higher in HLA-DQ-matched non-diabetic children in only 3 of 23 case controls ($p = 0.03$). In one comparison, neither patient nor HLA-DQ-matched control showed antibody binding to the 33-kDa wheat proteins.

Two-dimensional Western blots of wheat proteins probed with pooled sera from the same patients showed IgG antibody binding to several wheat proteins (Fig. 5, *panel C*). As in the case of diabetic BB rats, binding of antibodies to wheat proteins was widespread and more intense compared with controls (Fig. 5). Wheat storage globulin, Glb1, consists of two subunits with a molecular mass of 49 (pI 6.6) and 35 kDa (pI 6.9) (38). One of the proteins bound by antibodies from diabetic children (but not controls) displayed a mass of 50 kDa and pI of 6.5. When the nature of this protein was determined using LC-MS/MS, it was found to have peptides homologous to both Glb1 and WP5212. The expected (theoretical) peptide fragments of Glb1 and WP5212 and the experimental fragmentation detected by mass spectrometry are shown in Fig. 6.

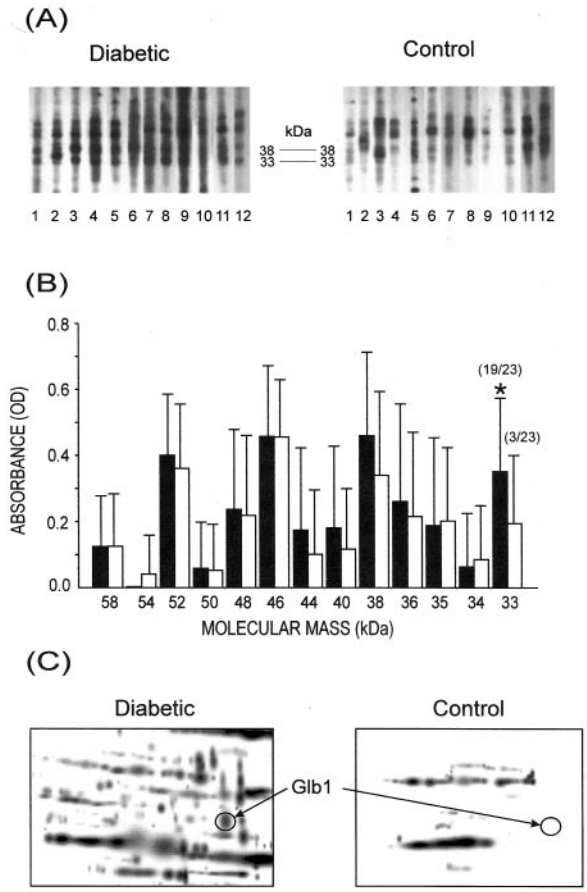

FIG. 5. **One-dimensional and two-dimensional Western analysis of antibody binding to wheat proteins in patients and HLA-DQ matched controls.** Examples of (*panel A*) one-dimensional Western blots of wheat proteins probed with serum samples from diabetic children and control children without diabetes. *Panel B*, the mean absorbance ± S.D. of (each) wheat protein band probed with serum from diabetic children (*filled bars*) and HLA-DQ-matched controls (*open bars*) (ANOVA/LSD; * indicates $p = 0.005$). *Panel C*, two-dimensional Western blot of wheat proteins probed with pooled serum samples from newly diagnosed diabetic children (*left*) or control children (*right*). Wheat storage globulin, Glb1, was bound by antibodies in serum from children with diabetes, but there was no binding using serum from non-diabetic controls.

DISCUSSION

When fed to diabetes-prone BB rats, diets in which wheat gluten was the sole protein source induced nearly three times as many cases of diabetes as a hydrolyzed casein-based diet (Fig. 1). To analyze as many potential diabetes-related wheat proteins as possible, we screened more than one million recombinant phage from a wheat cDNA expression library with pooled sera from diabetic rats. We isolated eight positive clones that were shown by nucleotide sequencing to contain three distinct sets of cDNA inserts. Of three representative clones, reactivity against WP5212 was strongest. BLAST searches revealed high similarity at the nucleotide and translated amino acid level with the wheat storage globulin protein, Glb1. IgG reactivity against Glb1 was strain-specific, highest in overt diabetic, lower in asymptomatic BB rats, and lowest in non-diabetes-prone BBc rats.

The autoimmune process involves progressive infiltration into the β-cell-containing core of the islets by mononuclear cells and macrophages, a process called insulitis. The severity and prevalence of insulitis or its sequelae (end stage islets) reflect the extent of damage in the pancreas. When sera from individual rats at different risks of developing diabetes were used, IgG reactivity against the Glb1 clone showed a remarkably close correlation with overall islet infiltration and damage (insulitis rating), as well as inflammation of individual islets (Fig. 3). The two other positive clones, WP12111 and WP23112, which shared amino acid homology with unidentified proteins from *A. thaliana* showed similar antibody reactivity to the control WP-CON clone (ascorbate peroxidase, *H. vulgare*), and there was no correlation between antibody reactivity and islet inflammation or damage (Fig. 2, *panel B*, and Fig. 3). These clones were not investigated further. These results demonstrated not only a strong immune reaction against the Glb1 protein in wheat-fed,

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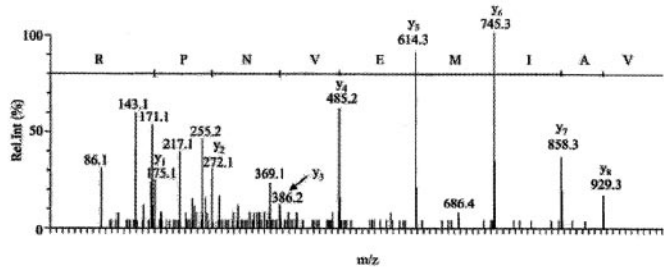
A



B

Theoretical fragmentation[a] of Glb1 (Acc. No. AAA34269)	Theoretical fragmentation[a] of WP5212	Experimental fragmentation
RPYVFGPR	**RPYVFGPR**	**RPYVFGPR**
VAIMEVNPR	**VAIMEVNPR**	**VAIMEVNPR**
AQDQDEGFVAGPEQQSR	ATIPLLFLLGTSLLFAAAVSASHDEEEDR	
FQFLSVKPLLASLSK	AFVVPGLTDADGVGYVAQGEGVLTVIENGEK	
GSESESEEEEEQQR	VAVANITPGSMTAPYLNTQSFK	
LGSPAQELTFGRPAR	QGDVIVAPAGSIMHLANTDGR	
DTFNLLEQRPK	LAVVLEGEGEVEIVCPHLGR	
SFHALANQDVR	GSAFVVPPGHPVVEIASSR	
GGHSLQQCVQR	DQQDEGFVAGPEQQQEHER	
ALRPFDQVSR	QASEGDQGHHWPLPPFR	
IIQSDHGFVR	GSSNLQVVCFEINAER	
HEQEEEQGR	LDDPAQELAFGRPAR	
GDEAVETFLR	FQYFSAKPLLASLSK	
EQEQEQER	GSGSESEEEQDQQR	
ILHTISVPGK	DTFNLLEQRPK	
EEEEDDQR	SFHALAQHDVR	
EAAEGGQGHR	GDEAVEAFLR	
DDQQQHGR	ALRPFDEVSR	
	ILHTISVPGK	
	GDSSTMATR	
	SEEEEDDR	
	DDQQQHGR	
	HEQEEQGR	
	DEEHGDGR	
	LGSLLGSR	
	LYEADAR	

[a] Theoretical fragmentation was calculated using the PeptideCutter program (Swiss Prot (2002) website address: http://us.expasy.org/tools/peptidecutter, Swiss Institute of Bioinformatics, Epalinges, Switzerland)

FIG. 6. **Identification of wheat storage globulin, Glb1, by in-gel tryptic digestion and capillary LC-MS/MS analysis.** *Panel A*, the MS/MS spectrum of the doubly protonated ion (MH_2^{2+}) at *m/z* 514.8 corresponding to the Glb1 tryptic peptide, VAIMEVNPR. The sequence of this peptide can be determined from the y ion series (*i.e.* fragment ions that originate from the carboxyl terminus of the peptide) as is indicated on the spectrum. *Panel B*, the theoretical and observed tryptic peptides of Glb1.

diabetes-prone BB rats but also a close link with the diabetogenic process in the target tissue.

Wheat gluten is a large macromolecular complex of polypeptides consisting mostly (80%) of gliadin and glutenin proteins that remain after repeated extraction of wheat flour with water, a process that removes most starch, albumins, and globulins. The endosperm of the growing wheat seed consists of starch granules embedded in a matrix composed mostly of storage proteins that provide nourishment and structure. Following two-dimensional electrophoresis, at least 1,300 endosperm proteins are visible (39). Traditionally, wheat proteins have been classified according to solubility; the major storage proteins (~80%) are the gliadins (soluble in aqueous alcohol) and glutenins (soluble in dilute acid or alkali), whereas albumins (water soluble) and globulins (salt-soluble) are minor constituents (~20%) (40). The classification by solubility does not clearly demarcate protein classes, and several proteins occur in more than one fraction. The complexity of the endosperm cell proteome, not only with respect to number but also with respect to size, physicochemical properties, and function, has made it difficult to identify specific diabetes-related proteins. Indeed, the wheat genome is estimated to be 16.5 gigabases, more than five times the size of the human genome.

Identifying Glb1, a salt-soluble globulin considered to be absent from wheat gluten, as a major diabetes-related protein was unexpected. However, wheat gluten proteins are difficult to separate into distinct fractions, and globulin proteins can remain trapped in the wheat gluten matrix (41). In the present study, Glb1 was identified in extracts of wheat gluten using two-dimensional Western blots and mass spectrometric analyses (Fig. 5, *panel C*, and Fig. 6). Thus, there are several possible interpretations of our findings: (i) Glb1 could be the main diabetes-related wheat protein; (ii) Glb1 is a normal component of wheat gluten; (iii) a diabetes-related antigenic structure in Glb1 is common among other wheat gluten proteins; and (iv) Glb1 is one diabetes-related protein among several candidates whose antigenicity or diabetogenicity may differ among wheat-induced diabetes cases. Considering these possibilities, we interpret our findings as follows. Glb1 is a normal trace component that becomes trapped in the wheat gluten protein matrix (41). It contains peptides that are highly antigenic in diabetes-prone BB rats fed wheat, and this immune reactivity closely parallels pancreatic damage. There was broad reactivity to wheat proteins in diabetes-prone BB rats and also in newly diagnosed, untreated diabetic patients, suggesting that abnormal reactivity to wheat is a common feature in diabetes-susceptible individuals. Our study indicates that of these wheat proteins, Glb1 was particularly antigenic and is a candidate diabetes-related protein.

Wheat proteins are related through structure and evolution to each other and also to other groups of seed proteins (42). The 2 S albumins and the α-amylase/trypsin inhibitors of cereals are part of the so-called prolamin superfamily (prolamins, 2 S albumins, and cereal inhibitors (globulins)) (43). These proteins form part of a fraction previously termed the chloroform/methanol-soluble fraction, and their removal from a wheat-

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based diet inhibited the development of diabetes in NOD mice (15). There are immunologically relevant structural similarities among the wheat proteins as shown by cross-reactivity of monoclonal antibodies between conserved epitopes in albumins and globulins (44). The present finding suggests that wheat-induced diabetes in BB rats may result at least in part from a misdirected immune reaction against non-gluten proteins that are co-isolated during the preparation of wheat gluten.

The prospective Western analysis showed a marked humoral response to certain low molecular mass (36 and 46 kDa) wheat proteins, particularly in animals that later developed overt diabetes (Fig. 4). These bands are similar in size to the 35- and 49-kDa subunits of Glb1 (45). Higher antibody binding to the 33-kDa band was present in 83% of diabetic children. This indicates a broad response to wheat proteins, one of which is Glb1. It is not yet clear whether reactivity to Glb1 is a specific response to a single diabetes-related protein or involves other wheat proteins. This broad immunoreactivity to wheat might reflect antigen spreading of the β-cell reactive process and unique individual patterns of abnormally high immune reactivity to wheat as reported in children with celiac disease (46).

Glb1 shares protein sequence homologies with an important immunomodulatory food protein, the peanut allergen, Ara h I. This member of the vicilin seed storage family is a major allergen in more than 90% of peanut-sensitive patients (47). The antibody-binding epitopes of the peanut allergen Ara h 1 have been mapped, and Glb1 shares homology with three of four immunodominant epitopes, and four of five other commonly recognized epitopes (37). This suggests common epitopes in both these immunomodulatory food proteins, a point that will require further analysis.

Globulins are the major protein constituent (90%) of soybean, a protein source that has been reported to promote the development of diabetes in BBdp rats, albeit to a lesser extent than the WP diet (2, 33). Furthermore, the Glb1 protein shares sequence homology with a soybean glycinin protein, suggesting that wheat and soybean might have common immunogenic and possibly diabetogenic proteins. Further studies are needed to clarify if these proteins are related to diabetes.

Sequence homologies were also observed between Glb1 and tight junction protein 2, which is part of a complex of proteins that controls the permeability of the intestinal epithelium. This is of particular interest because abnormally increased gut permeability to mannitol, a marker of paracellular transport, has been reported in pre-diabetic BB rats (29) and newly diagnosed patients with type 1 diabetes (30). Cross-reactivity between Glb1 and tight junction proteins might be expected to damage the gut mucosa of BBdp rats making it more permeable to dietary antigens, possibly overwhelming the normal oral tolerance mechanisms, and leading to increased antibody production against dietary wheat proteins.

Two-dimensional blots also showed higher antibody binding in diabetic children to several other as yet unidentified wheat proteins (Fig. 5, *panels A* and *C*). Glb1 was among these proteins but absent in the two-dimensional blots probed with control serum in keeping with the result of the one-dimensional analysis (Fig. 5, *panel A*). Our results support the interpretation that diabetic patients have unique patterns of immune reactivity, some of which include Glb1. Increased peripheral blood T-cell reactivity to wheat proteins was seen in 24% of newly diagnosed patients with type 1 diabetes, compared with only 5% of non-diabetic controls (22). Taken together, these data are consistent with the proposition that wheat antigens are the target of inappropriate immune responses in certain individuals who are genetically susceptible to develop autoimmune diabetes.

In patients with type 1 diabetes, the presence of autoantibodies to either glutamic acid decarboxylase or islet antigen-2 has been shown to be closely correlated with *in situ* pancreatic islet inflammation (insulitis) and/or hyperexpression of major histocompatibility complex class I antigens in islets (23). Similarly, antibodies from BBdp and diabetic rats showed strong reactivity to the Glb1 protein, and this immunoreactivity correlated closely with the destructive immune process that targets the pancreatic islet β-cells in the pancreas. The close correlation between antibody reactivity to Glb1 and islet inflammation in BB diabetes-prone and diabetic rats represents a new association between a previously unidentified wheat antigen and the target tissue. The fact that higher immunoreactivity to Glb1 was observed in patients compared with HLA-DQ matched non-diabetic children raises the possibility that wheat may also be involved in the pathogenesis of human type 1 diabetes.

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