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

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

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 BB1 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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patients 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.

**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 EcoRI/Blunt 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 × 10⁴ 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 × 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, Fcy 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 MgCl₂) containing nitro blue tetrazolium chloride (0.3 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (Promega). Wax and destaining were performed using 5% acetic acid/70% methanol. Secondary antibody density was quantified by densitometry of autoradiographs using the ImageQuant™ software package (Molecular Dynamics, Sunnyvale, CA).

**Identification and characterization of clones isolated from a wheat cDNA expression library** (Table I)

- **WP5212** Triticum aestivum (wheat) storage protein (Gib 1) gene, 90%/1387
  - **WP12111** Clone CNW03PL453 ITCC CNW wheat powdery mildew-resistant line, T. aestivum, 96%/138
  - **WP23112** Arabidopsis thaliana DNA chromosome 3, BAC clone T16L24, 96%/542
  - **WPCON** Ascorbate peroxidase (Hordeum vulgare) 91%/526

**Table I**

<table>
<thead>
<tr>
<th>Clone</th>
<th>DNA homology, % identity/no. bp</th>
<th>Amino acid homology, % identity/no. amino acids</th>
<th>ORF length</th>
<th>Putative protein length (amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WP5212 T. aestivum (wheat) storage protein (Gib 1) gene, 90%/1387</td>
<td>T. aestivum (wheat) storage protein, 80%/542</td>
<td>1890</td>
<td>629</td>
<td></td>
</tr>
<tr>
<td>WP12111 Clone CNW03PL453 ITCC CNW wheat powdery mildew-resistant line, T. aestivum, 96%/138</td>
<td>Unknown protein, A. thaliana, 63%/144</td>
<td>789</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td>WP23112 Arabidopsis thaliana DNA chromosome 3, BAC clone T16L24, 96%/542</td>
<td>Putative protein, A. thaliana, 62%/150</td>
<td>624</td>
<td>207</td>
<td></td>
</tr>
<tr>
<td>WPCON Ascorbate peroxidase (Hordeum vulgare) 91%/526</td>
<td>Ascorbate peroxidase (H. vulgare), 96%/86</td>
<td>366</td>
<td>121</td>
<td></td>
</tr>
</tbody>
</table>

**a** BLASTx and BLASTx sequence homology searches were performed using the GenBank™ and TIGR Wheat Gene Index databases. **b** The open reading frame contains 95 bp from the 5’ β-galactosidase gene. **c** Determined using Clone Manager™ (Sci-Ed Software (2002) website address: www.scied.com/ses_cm6.htm, Scientific and Educational Software, Durham, NC).
TABLE II
Proteins with sequence homologies to Gi1 found by BLAST (28) searches of the GenBank™-2 and NCBI human genome data bases

<table>
<thead>
<tr>
<th>Group</th>
<th>Homologous protein</th>
<th>Expect value</th>
<th>Amino acid homology, % identity/aa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Allergen Ara h 1, clone P17 precursor (Ara h 1)</td>
<td>4e⁻²⁵</td>
<td>25%/630</td>
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<tr>
<td></td>
<td>Ara h 1 commonly recognized epitope ³</td>
<td>7.7 × 10⁵</td>
<td>60%/5</td>
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<tr>
<td></td>
<td>Ara h 1 immunodominant epitope ³</td>
<td>7.7 × 10⁵</td>
<td>50%/6</td>
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<tr>
<td></td>
<td>Ara h 1 commonly recognized epitope ⁴</td>
<td>5.4 × 10⁴</td>
<td>100%/4</td>
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<td></td>
<td>Ara h 1 commonly recognized epitope ⁵</td>
<td>4.3 × 10⁶</td>
<td>80%/5</td>
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<td></td>
<td>Ara h 1 commonly recognized epitope ⁶</td>
<td>5.4 × 10⁴</td>
<td>83%/5</td>
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<td>Ara h 1 commonly recognized epitope ⁷</td>
<td>8.9 × 10²</td>
<td>70%/10</td>
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<tr>
<td></td>
<td>Ara h 1 immunodominant epitope ⁸</td>
<td>7.7 × 10⁵</td>
<td>80%/5</td>
</tr>
<tr>
<td></td>
<td>Allergen Gly m Bd 28K (Glycine max)</td>
<td>&lt;0.005</td>
<td>21%/483</td>
</tr>
<tr>
<td></td>
<td>Gi</td>
<td>12687782</td>
<td>dbj</td>
</tr>
<tr>
<td>2</td>
<td>Root allergen protein (RAP), dandelion</td>
<td>&lt;888</td>
<td>31%/58</td>
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<tr>
<td></td>
<td>Gi</td>
<td>7388038</td>
<td>ap</td>
</tr>
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<td>3</td>
<td>Tight junction protein, ZO-2, chicken</td>
<td>&lt;17</td>
<td>29%/124</td>
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<td></td>
<td>Gi</td>
<td>7512238</td>
<td>pl</td>
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<td></td>
<td>Similar to tight junction protein ZO-1 (Homo sapiens)</td>
<td>&lt;1.2</td>
<td>28%/14</td>
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<tr>
<td></td>
<td>Gi</td>
<td>17436387</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tight junction protein 2 (Zona occludens 2); Friedreich</td>
<td>&lt;5.8</td>
<td>31%/133</td>
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<tr>
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<td>ataxia region gene X104 (Tight junction protein ZO-2) (H. sapiens)</td>
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<td>4759342</td>
</tr>
<tr>
<td></td>
<td>Similar to Tight junction protein ZO-2 (Z. occludens 2 protein) (Tight junction protein 2)(H. sapiens)</td>
<td>&lt;5.8</td>
<td>31%/133</td>
</tr>
<tr>
<td></td>
<td>Gi</td>
<td>13639591</td>
<td></td>
</tr>
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</table>

¹ 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.

² Homology achieved using the sequence homology matrix-PAM-30, recommended for comparing sequences less than 35 amino acids in length.

³ 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
nuclear cells. The mean of 10 islets per animal was used for an overall insulin score. Immunization 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 (Solkar-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% niacin, 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 386 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 AAL08491.1).

**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 (developed overt diabetes before 120 days, open bars), asymptomatic BBc (no clinical symptoms of diabetes by 120 days, cross-hatched bars), or control (hatched bars), asymptomatic (n = 10), BB rats with positive antibody reactivity to the wheat proteins is shown. A positive antibody level was represented by an antibody level that was significantly different (p < 0.05) from that of the asymptomatic and control rats (open bars). Panel B, mean antibody reactivities (intensity/pixel) ± S.D. to the recombinant wheat proteins screened with diabetic (n = 7), asymptomatic (n = 10), or control rats (n = 9), 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), asymptomatic (n = 10), or control (n = 9) rats. BB rats are shown. Individual values for diabetic (diagonals), asymptomatic (squares), and 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 ≤ 0.02; * indicates significant versus asymptomatic rats, p ≤ 0.02).
**RESULTS**

**Wheat Protein Diets Can Modulate Diabetes Outcome**—Animals fed a non-purified, defined, mainly wheat-based diet (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 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 × 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™ and TIGR Wheat Gene Index™ 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 AAA42969.1), Glb1.

WP5212 also shared sequence homology 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 four of five other commonly recognized epitopes (37). WP5212 also had sequence homology with two other plant allergens, a soybean protein (*Glycin max*, GenBank™ accession number BAB21619) and a dandelion root protein (*Taraxacam 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 4799342, 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).

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 product (Table I).
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.
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^{-5}) and mean insulitis score (Fig. 3, panel B, r = 0.78, p = 3 \times 10^{-3}). 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 1600 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 6.5). 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.

**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,
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 2S albumins and the a-amylase/trypsin inhibitors of cereals are part of the so-called prolamin superfamily (prolamins, 2S 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-
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 against 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 I 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 glycumin 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 mannanot, 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 processes 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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