Identification of Peptides From Autoantigens GAD65 and IA-2 That Bind to HLA Class II Molecules Predisposing to or Protecting From Type 1 Diabetes

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Type 1 diabetes is a T-cell–mediated disease in which presentation of autoantigens to CD4+ T-cells is thought to play a crucial role. Polymorphism of HLA class II genes accounts for 50% of the genetic risk of contracting type 1 diabetes. HLA-DQ and -DR molecules predisposing to or protecting from type 1 diabetes have been identified, but the molecular basis controlling these associations is as yet undefined. Apart from distinct thymic selection of autoreactive T-cells by susceptible and protective HLA molecules, exclusive presentation of autoantigenic peptides by type 1 diabetes–predisposing HLA molecules or, alternatively, induction of regulatory T-cells by protective alleles are potential mechanisms for modification of type 1 diabetes risk by HLA polymorphism. As a first step in exploring the role of HLA molecules in autoantigen-specific cellular responses in type 1 diabetes, we have screened peptides covering the sequence of two major autoantigens targeted by humoral and cellular immune responses, GAD65 and islet associated-2 (IA-2), for binding to class II molecules. We developed a sensitive novel competition binding assay allowing us to measure peptide binding on intact cells to 10 HLA-DR and 4 HLA-DQ molecules. For all tested alleles, multiple peptides binding with high affinity were identified. We report clustering of binding peptides in the COOH-terminal regions of GAD65 and IA-2, as well as highly promiscuous binding patterns of some peptides. Our results demonstrate that most peptides derived from the GAD and IA-2 autoantigens can bind to both type 1 diabetes–predisposing and type 1 diabetes–protective HLA molecules, although some exceptions were observed. The binding inventory presented here for GAD and IA-2 peptides can be useful for mapping natural epitopes and predicting peptide-specific responses induced by preventive immunization. *Diabetes* 48:1937–1947, 1999

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BLCL, B-lymphoblastoid cell line; EBV, Epstein-Barr virus; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; IA-2, islet associated-2; IA-2ic, intracellular IA-2; IC_{50}, 50% inhibitory concentration; mAb, monoclonal antibody; MBP, myelin basic protein; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; Th, T-helper.

Type 1 diabetes results from a T-cell–mediated destruction of insulin-producing β-cells of the islets of Langerhans in genetically predisposed individuals (1). The major histocompatibility complex (MHC) region is by far the most important region conferring susceptibility to type 1 diabetes because it contributes ~50% of the disease risk (2). Several type 1 diabetes–associated HLA class II haplotypes have been identified in various ethnic groups. Susceptibility is associated primarily with the DRB1*03-DQB1*0201 and DRB1*04-DQB1*0302 haplotypes. Conversely, a strong protection is conferred by the DRB1*1501-DQB1*0602 haplotype (3–5). A predominant role has usually been attributed to the DQ-encoded molecules on these haplotypes: protection on the DR15 haplotype associates most closely with the DQB1*0602 molecule, and among type 1 diabetes–predisposing alleles, DQB1*0302 has the strongest effect. However, an independent contribution of the DR locus is now generally admitted, as exemplified on the various DRB1*04-DQB1*0302 haplotypes. Thus, DRB1*0402, DRB1*0405, and, to a lesser extent, DRB1*0401 increase the disease risk conferred by the predisposing DQB1*0302 allele, whereas DRB1*0403, DRB1*0404, DRB1*0406, and DRB1*0407 antagonize its effect and confer a dominant protection (5–9). In addition, two phenomenons deserve particular attention: the clear synergistic effect between the predisposing DR3 and DR4 haplotypes and the fact that protective alleles exert a dominant effect over predisposition in individuals bearing both types of alleles.

MHC class II molecules present short peptides derived from self or foreign protein antigens to CD4+ helper T-cells. The extreme degree of polymorphism within the peptide-binding groove of HLA class II molecules leads to differential binding affinity of peptides for distinct alleles. The crystal structure of a DR molecule complexed with a single peptide has greatly enhanced our understanding of the factors governing how peptides are bound by class II molecules (10). Specific pockets of the MHC molecule accommodate the side chains of anchor residues in the peptide, generally located in relative positions 1, 4, 6, 7, and 9, and determine the peptide-binding specificity. Through sequence analyses of naturally processed eluted peptides or binding studies with synthetic peptides (11), peptide-binding motifs for the majority of DR and DQ alleles associated with predisposition to or protection from type 1 diabetes have now been described (DRB1*0301 [12], DRB1*0401

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[13–16], DRB1*0402 [13,17], DRB1*0404 [11], DRB1*0405 [13,18], DRB1*0406 [18], DRB1*1501 [19], DRB5*0101 [13,19], DRB4*0101 [20], DQB1*0301 [21,22], DQB1*0302 [22–24], DQB1*0201 [25–28], and DQB1*0602 [29]).

It is likely that the allelic polymorphisms that are responsible for determining specific peptide binding also underlie the MHC contribution to type 1 diabetes. However, the exact mechanism by which different HLA class II molecules predispose to or protect from type 1 diabetes is still a mystery. A first but unlikely hypothesis is that predisposing molecules can bind autoantigenic peptides whereas protective alleles cannot. Another hypothesis postulates that protective molecules mediate the intrathymic deletion of diabetogenic autoreactive T-cell clones (30) or the positive selection of regulatory cells (31) more efficiently than predisposing molecules do. Alternatively, class II alleles may present autoantigenic peptides to functionally different T-cell subsets and induce a diversion of the phenotype of diabetogenic T-cells (32,33), with predisposing alleles driving a T-helper (Th)1 (cellular) destructive response whereas protective alleles stimulate a Th2 response. Finally, the hypothesis of determinant capture proposes that protective and predisposing alleles can compete for binding of overlapping autoantigenic peptides recognized by diabetogenic T-cells (34,35).

Thus far, although many experimental data provide support for or against these different possibilities, the involved mechanism remains obscure. Furthermore, the nature of autoantigenic peptides presented by class II molecules to diabetogenic T-cells is not clear. More than a dozen autoantigens associated with pancreatic islet cells have been reported to function as targets of antibody or T-cell responses in type 1 diabetic patients (1). Among them, two enzymes expressed in neuroendocrine cells—65-kDa GAD (GAD65) and the tyrosine phosphatase IA-2—appear to play a predominant role and have been identified as autoantigens because of the presence of early autoantibodies in prediabetic subjects and recent-onset patients (36–43). Furthermore, proliferative T-cell responses to GAD and IA-2 are found in prediabetic individuals (44–48).

To get further insights into the mechanisms underlying type 1 diabetes risk modification by HLA class II polymorphism, we have developed a competitive peptide-binding assay on intact cells, allowing us to screen synthetic peptide ligands derived from GAD65 and islet associated-2 (IA-2) for binding to HLA-DR and -DQ type 1 diabetes–associated alleles.

RESEARCH DESIGN AND METHODS

Cell lines. The following Epstein-Barr virus (EBV)-transformed homozygous B-lymphoblastoid cell lines (BLCLs) were used: EA (DRB1*1501-DRB5*0101-DQA1*0102-DQB1*0602), DUCAF (DRB1*0301-DRB3*0101-DQA1*0501-DQB1*0201), MLF (DRB1*0401-DRB4*0101-DQA1*0301-DQB1*0301), YAR (DRB1*0402-DRB4*0101-DQA1*0301-DQB1*0302), PE117 (DRB1*0404-DRB4*0101-DQA1*0301-DQB1*0302), and LKT3 (DRB1*0405-DRB4*0101-DQA1*0301-DQB1*0401), all described in the Tenth and Eleventh International Histocompatibility Workshops. Cells were grown in RPMI 1640 with L-glutamine, supplemented with 10% fetal calf serum (FCS) and 1 mmol/l sodium pyruvate.

The following HLA class II (DRA and DRB1) L-cell transfectants were purchased from European Collection of Biomedical Research (ECBR) (Genova, Italy): DAP3-DR4 (DRB1*0401), 2164.11 (DRB1*0402), 9.0.4.C43 (DRB1*0403), L165.6 (DRB1*0404), L166.1 (DRB1*0405), LDR+25 (DRB1*0406), L-DRW53-DW15 (DRB4*0101), LR3.1/3 (DRB1*0301), and DAP3-DR2a (DRB5*0101). BLS-DRB1*1501 transfectants were a gift from G. Nepom (Seattle, WA). Transfectants were grown in Dulbecco's modified Eagle's medium glutamate with 10% FCS and 1 mmol/l sodium pyruvate and in the presence of the selecting reagents recommended by NCBR.

All class II molecules are designated using the official nomenclature for DRB- and DQB-encoded alleles. Because of the very low DRA polymorphism and the absolute linkage disequilibrium that exists in Caucasians between the DQA and DQB genes, the DRA- and DQA-encoded alleles are not specified (for instance DRB1*0401 is used for the DR4 molecule encoded by DRA*0101 and DRB1*0401, and DQB1*0302 is used for the DQ8 molecule encoded by DQA1*0301 and DQB1*0302).

Antibodies. Mouse hybridoma L243 secreting a monoclonal antibody (mAb) to HLA class II DR molecules was purchased from the American Type Culture Collection (ATCC, Rockville, MD). SPV-L3 (anti-DQ) hybridoma was kindly provided by H. Spits (Amsterdam, the Netherlands).

SPV-L3 and L243 mAbs were purified from ascites using protein A-sepharose chromatography.

Peptides. Peptides were synthesized on 9-fluorenylmethylcarbonyl (Fmoc) amino acid (Wang resin [GAD peptides]) or TCP resin (IA-2 peptides; PepChem, Tübingen, Germany) using a robot system for simultaneous multiple solid-phase synthesis (SMPS 350; Zeisser, Frankfurt, Germany). The purity of all peptides was checked by reversed-phase high-performance liquid chromatography (HPLC). Identity of peptides was confirmed by electrospray mass spectrometry (API III triple quadruple ion-spray mass spectrometer; PE Sciex, Thornhill, Canada). If nec-

TABLE 1
HLA-DR and -DQ binding reporter peptides

Reporter peptide			
Name	Sequence	Allele(s) bound	IC_{50} (μmol/l)
B2–180† (HA Y307–319)	YPKFVKQNTLKAA	DRB1*0401	3
		DRB1*0402	2.2
		DRB1*0404	2.5
		DRB1*0405	2.1
B2–181	GIRAAYAAAA	DRB1*1501	NA
B2–182 (MBP 83–97)	ENPVVHFFKNIVTPR	DRB5*0101	NA
B2–183	IAYDAAAAA	DQB1*0301	29.7
		DQB1*0302	16
		DRB1*0301	NA
B2–185 (MB 243–255)	KPLLIIAEDVEGEY	DQB1*0201	3.6
B2–186 (λR 12–24)	LEDARRLKIYEK	DQB1*0302	NA
B2–187 (AYK)	AAYAAAAAAKAA	DQB1*0301	28
		DQB1*0602	47
B-0802† (CLIP)	LPKPPKPVSK RMAFPLLMQALPMG	DRB4*0101	NA
		DRB1*0403	
		DRB1*0406	

†Used at 1 μmol/l; all others used at 3 μmol/l. NA, nonbiotinylated reporter peptide not available for competition experiment.

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essary, peptides were further purified by preparative HPLC to a purity of at least 95%. To biotinylate reporter peptides, resin-bound peptides were first coupled to a NH_2-terminal spacer composed of εLys-βAla-εAha-βAla and then to a fivefold molar excess of D-biotin. Table 1 lists the sequences of the biotinylated reporter peptides used for individual HLA class II molecules.

For the GAD65 protein, a set of 57 peptides spanning the entire 585–amino acid sequence were synthesized. Among them, 44 were 15-mer peptides with overlaps of 5 amino acids, and 13 were 20-mer peptides with overlaps of 10 amino acids.

For the IA-2 protein, we used a set of 37 20-mer peptides with overlaps of 10 amino acids, spanning the intracytoplasmic part of the protein (amino acids 601–980). Although IA-2 has a signal peptide, an extracellular domain, a transmembrane region, and an intracellular domain, the protein is cleaved within its ectodomain close to neurosecretory granule membranes, so that very little of the extracellular domain is present on the surface of pancreatic β-cells. Treatment of the full-length IA-2 molecule or the intracellular domain with trypsin results in a fragment of 40 kDa to which type 1 diabetic patients have autoantibodies. IA-2–specific β-cell epitopes are contained within the juxta-membrane (amino acids 601–682) and the tyrosine phosphatase domain (amino acids 687–979) and not within the extracellular domain of IA-2 (43). Finally, peripheral blood mononuclear cells from recent-onset type 1 diabetic patients proliferate to the internal domain of the IA-2 protein (48).

Whole cell peptide-binding assay. A whole cell competitive peptide-binding assay was developed by modification of a previously described direct binding assay (49,50). Briefly, 2×10^6 cells (BLCLs or transfectants) per sample were fixed in 0.5% paraformaldehyde for 10 min, washed once in Hanks' balanced salt solution (HBSS)-1% FCS, then extensively in HBSS, and incubated with 1–3 µmol/l of the appropriate biotinylated reporter peptide in 100 µl of 150 mmol/l citrate-phosphate buffer, pH 4.4 (or pH 6.5 for DQB1*0301), and 1 mmol/l phenylmethylsulfonyl fluoride for 2 h at 37°C in the absence or presence of various concentrations (0.1–300 µmol/l) of the nonbiotinylated competitor peptide. Cells were washed twice in HBSS to remove unbound peptide and lysed in 100 µl of buffer containing 0.5% Nonidet P-40, 150 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 7.4, and protease inhibitors (0.1 mg/ml 4-(2-aminoethyl)-benzenesulfonylfluoride-HCl, 0.4 mg/ml EDTA, 1 µg/ml aprotinin, 0.5 µg/ml pepstatin, 0.5 µg/ml leupeptin, 1 µmol/l benzamidin) for 30 min at 4°C. The lysates were cleared by centrifugation and transferred to 96-well plates precoated with 10 µg/ml of the appropriate mAb (L243 for capture of DR or SPV-L3 for capture of DQ) and blocked with phosphate-buffered saline (PBS) containing 0.02% Tween 20 and 2% bovine serum albumin. Then, 100 µl of 50 mmol/l Tris-HCl, pH 7.15, containing 0.02% dodecyl β-D-maltoside (for DR) or 0.75% *n*-octyl β-D-glucopyranoside (for DQ) were added, and lysates were incubated overnight at 4°C. The wells were washed six times with PBS-0.02% Tween 20, and europium-labeled streptavidin (Wallac, Gaithersburg, MD) was added for 4 h at 4°C. The plate was washed six times with PBS-0.02% Tween 20, and room temperature enhancement buffer (Wallac) was added for 5 min. Fluorescence was measured in a Delfia 1232 fluorometer (Wallac).

The concentration corresponding to 50% inhibition of reporter peptide binding (IC_{50}) was determined by plotting fluorescent signals obtained for increasing concentrations of each competitor peptide on semi-logarithmic paper. Relative binding values were calculated by dividing the IC_{50} value by the molar concentration of the reporter peptide used in the assay.

RESULTS

Competitive peptide-binding assay on intact cells. Binding of GAD65 and IA-2 peptides to DR and DQ molecules was analyzed using a modified peptide-binding assay. Briefly, a biotinylated reporter peptide was incubated for a relatively short time with paraformaldehyde-fixed cells expressing the appropriate HLA class II molecules in the absence or presence of nonbiotinylated competitor peptide before HLA class II molecules were solubilized and captured by HLA-DR– or -DQ–specific mAbs coated onto polystyrene microwells. Bound peptide was measured using europium-tagged streptavidin. Efficient detection of binding competition by autoantigen-derived peptides in this assay correlated with several parameters.

First, satisfactory assay sensitivity depended on identification of suitable reporter peptides that bound with good signal-to-noise ratio to their respective class II molecules (Table 1). Reporter peptides were selected from peptides characterized previously for binding to HLA-DR or -DQ molecules (14,25,49,51). A peptide corresponding to the class II invariant chain-associated peptide (CLIP) sequence 97–121 of the invariant chain, which binds promiscuously to most HLA class II alleles, was used when no other efficient reporter peptide was available (52). Allele specificity of the reporter peptides was determined by testing on various HLA-DR and -DQ molecules. Specificity of binding to cell-bound DR or DQ molecules was confirmed by using the nonrelevant anti-DQ or anti-DR capturing antibody (Fig. 1*A*). Some reporter peptides bound to several class II alleles (e.g., B2–180 was used for different DR4 subtypes), whereas others were highly allele-specific (e.g., B2–186 was specific for DQB1*0302). Peptide saturation curves were determined for each reporter peptide, allowing to define the concentration that was further used for competition experiments. The affinity of the different reporter peptides were determined in competitive inhibition experiments with the corresponding nonbiotinylated reporter peptide when available (Table 1).

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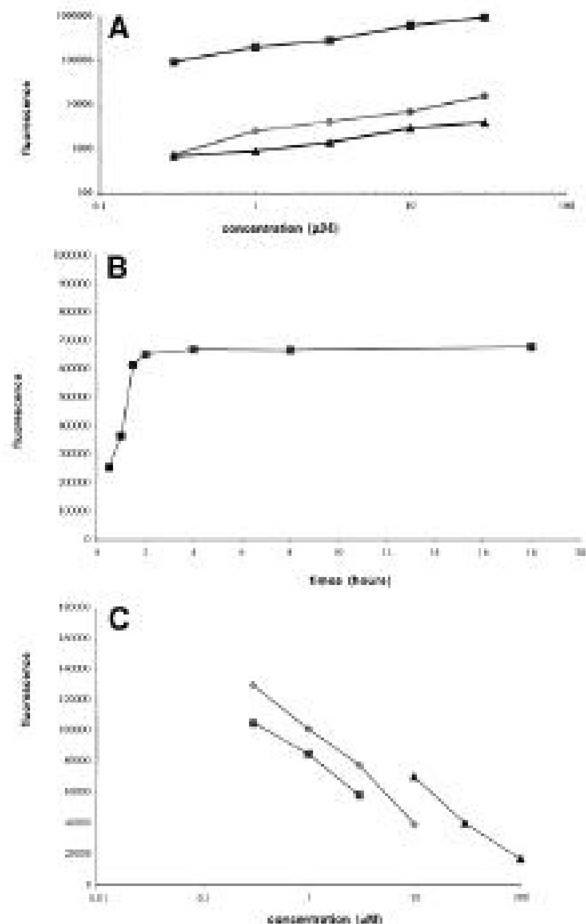

FIG. 1. Peptide binding to paraformaldehyde-fixed cells. *A*: Binding of biotinylated peptide B2–180 to MLF lymphoblastoid EBV-transformed B-cells expressing the DRB1*0401 and DQB1*0301 molecules. Biotinylated peptide (0.5–30 µmol/l) was incubated with cells for 2 h at 37°C. mAb L243 was used to capture DRB1*0401 molecules (■), and SPV-L3 was used to capture DQB1*0301 molecules (●). Negative control values represent binding of B2–180 to the HLA class II–negative control line T2 (incubation with L243-coated plates, ▲). *B*: Kinetics of binding of the B2–180 reporter peptide to DRB1*0401 cells. Biotinylated peptide B2–180 (1 µmol/l) was incubated with MLF lymphoblastoid B-cells (DRB1*0401) for 0.5–18 h at 37°C. *C*: Competition for binding to DRB1*0404 cells of 1 µmol/l biotinylated peptide B2–180 with various concentrations of nonbiotinylated peptides B2–180 (■) or I951–970 (◆) and I921–940 (▲).

The second critical parameter for assay sensitivity was the duration of cell incubation with reporter peptides. A maximal binding of the reporter peptide was obtained very rapidly after 2 h of incubation (Fig. 1*B*). However, competitive inhibition with the nonbiotinylated reporter peptide or with a panel of unrelated peptides could not be detected consistently when long incubations (>2 h) were performed. Instead, a nonspecific increase in the binding of the biotinylated peptide was noted in the presence of competitor peptide when cells were incubated for long periods simultaneously or sequentially with competitor plus reporter peptide. Thus, optimal conditions for competition binding assays using entire cells varied from assays with purified MHC class II molecules, which typically require a long incubation time of >48 h. In all further competition experiments, the reporter peptide (1–3 µmol/l) was therefore incubated together with various concentrations of competitor peptide (0.1–100 molar excess) for 2 h before solubilization and capture of class II molecules (Fig. 1*C*).

Binding of biotinylated peptides was pH dependent, as previously described (49,53). Maximal binding was observed at low pH (pH 4.4), except for the DQB1*0301 allele, where optimal binding was observed at pH 6.5. Optimal binding at low pH for class II proteins expressed in their normal cellular membrane environment is consistent with the fact that peptide loading occurs in acidic endosomal compartments under physiological conditions. Finally, peptide binding also depended on the cellular growth phase. Inter-assay reproducibility required cells in the exponential phase. All experiments were repeated at least three times, and in some cases, up to five times. Results are thus given as IC_{50} ± SD.

Some HLA-DR haplotypes carry more than one DRB gene and therefore express several DR molecules; for example, DRB4 is expressed in $DR4^+$ cells, whereas the DRB5 product is expressed in DR15 cells. Because reporter peptide may bind to both DR molecules carried in a haplotype, it was important to determine the contribution of the different DRB products on such haplotypes to assay results by using fibroblast transfectants that express only one type of DR molecule. Transfectants were therefore used exclusively or in addition to BLCLs to study binding to HLA-DR molecules encoded in DR4 and DR15 haplotypes.

Binding of GAD65-derived peptides to DR and DQ molecules

Binding affinity. For all tested HLA class II molecules, some peptides inhibited the binding completely at a low concentration (IC_{50} 1–10 µmol/l), some inhibited the binding more weakly (10 µmol/l < IC_{50} < 100 µmol/l), and some did not inhibit the binding at all (IC_{50} unmeasurable). Each of the 14 DR or DQ alleles tested bound at least one GAD65-derived peptide efficiently (IC_{50} <100 µmol/l), except DRB4*0101, which only bound peptides with IC_{50} >135 µmol/l. Alleles expressed on the DR3 haplotype were the most selective: the DQB1*0201 (DQ2) allele bound only one peptide (G176–195), and the DRB1*0301 (DR3) allele bound two peptides (G301–315 and G511–525), which is in agreement with recent data showing that all GAD65 peptides except these two bound very poorly or not at all to the DR3 molecule (54). However, for GAD65 peptides, we did not confirm a previous suggestion that DQ2 is a more promiscuous peptide binder than DR3 (25). All other DR or DQ alleles tested bound more than five peptides, and in some cases, up to nine. The mean number of peptides with high/intermediate affinity binding to predisposing and protective alleles was similar (six in both cases). These results are shown in Fig. 2.

Although many peptides were able to bind to both DR and DQ molecules, a limited number of peptides showed either DR-specific (G241–255, G266–285, G511–525, G556–575) or DQ-specific interactions (G331–345, G401–415). Because the absolute binding affinities of individual reporter peptides for the respective HLA class II alleles varied, a direct comparison of competitor peptide affinities for DR and DQ molecules was not possible. However, the range of binding affinities for both types of HLA class II molecules appeared similar. In the same way, the binding affinities of GAD65 peptides for predisposing alleles were comparable to those for protective alleles.

Peptides from the different regions of the GAD65 protein could bind to different DR and DQ alleles. However, peptides derived from the NH_2-terminal part of the protein (amino acids 1–155) bound only to protective alleles: G21–35 and G41–55 bound exclusively to DQB1*0602 (DQ6), G66–85 to DRB1*0403, and G141–155 to DRB1*1501 (DR15).

Allele-specific versus promiscuous binding. A limited number of peptides showed allele-specific interactions. Apart from the above-mentioned NH_2-terminal peptides that bound to protective alleles, these included G221–235 binding to DRB1*1501, G361–375 binding to DQB1*0301, and G486–505 binding to DRB1*0405.

Other peptides, although not strictly allele-specific, displayed a preference for a small number of molecules, and interestingly such molecules belonged to the same category of alleles, i.e., protective or susceptible. For instance, G191–205 bound to the protective DQB1*0301, DRB1*0403, and DRB1*0406 alleles; G241–255 bound to the protective DRB1*1501, DRB5*0101, and DRB1*0406 alleles; and G501–515 bound to the protective DQB1*0602 and DRB5*0101 alleles, whereas G431–445 bound to the predisposing DRB1*0405 and DQB1*0302 molecules. Two peptides bound in a DQ-specific way: G331–345 bound to DQB1*0301 and DQB1*0602 (both protective), and G401–415 bound to DQB1*0302 and DQB1*0602 (the former predisposing and the latter protective).

By contrast, other peptides (G176–195, G246–265, G511–525, and G555–575) bound promiscuously to many alleles. Such alleles usually encompassed the group of DR4 subtypes, as well as DRB1*1501, DRB5*0101, and a few DQ alleles. This was particularly striking in the case of the two COOH-terminal peptides G511–525 and G556–575, which bound very efficiently to most DR alleles. Another promiscuous region corresponds to the G246–265 peptide. This region has already been extensively analyzed because the GAD65 sequence 253–265 (IARFKMFPEVKEK) is highly homologous to the P2-C sequence of the Coxsackie virus, which has been epidemiologically associated with type 1 diabetes. This observation has led to the hypothesis that molecular mimicry with exogenous pathogens might precipitate type 1 diabetes (47,55). As previously reported by Kwok et al. (22), this peptide bound with high affinity to DQB1*0302, and more weakly to DQB1*0602, but not at all to DQB1*0201 and DQB1*0301. However, we found that it also bound efficiently to the DRB1*0401, 0402, and 0405 subtypes and with very high affinity to DRB5*0101 (IC_{50} 3 µmol/l). Interestingly, the two adjacent peptides, G241–255 and G266–285, bound

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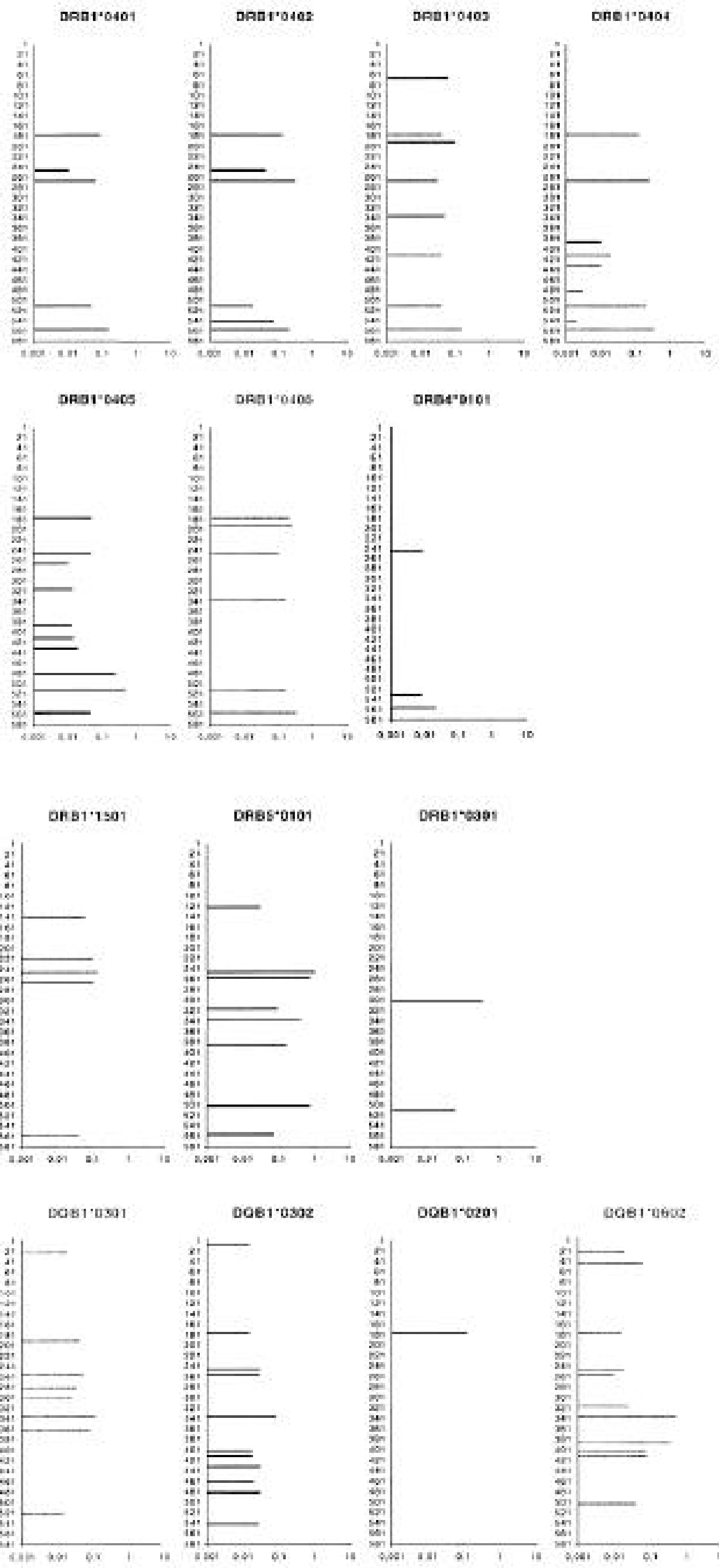

FIG. 2. Binding of GAD65 peptides to HLA-DR and -DQ alleles. Biotinylated reporter peptide (1–3 μmol/l) was incubated for 2 h with 2 × 10^6 paraformaldehyde-fixed cells in the absence or presence of various concentrations (0.1–100 molar excess) of GAD65 competitor peptides. Cells were washed to remove unbound peptide and lysed. HLA-DR or -DQ molecules were captured on a microtiter plate coated with L243 or SPV-L3 mAb, respectively. Bound biotinylated peptide was detected by fluorescence, using europium-labeled streptavidin. IC_{50} values represent the average of two to four determinations per peptide. IC_{50} values differed generally by a factor of less than two between individual experiments. Bars indicate the location of the NH_2-terminals of 15- or 20-mer peptides with high or intermediate binding affinity. Results are expressed as the inverse of the ratio of the IC_{50} value to the concentration of the assay-specific reporter peptide (1/normalized IC_{50}).

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with a very high affinity to DRB1*1501/DRB5*0101/DRB1*0406 and to DRB1*0401/0402/0403/0404/DRB1*1501, respectively. Thus, the region spanning amino acids 241–285 is able to bind to the vast majority of DR or DQ predisposing and protective alleles, with a strong preference for DRB5*0101, which is expressed on the DR15-DQ6 protective haplotype. It must be emphasized that G246–265 also corresponds to a DRB5*0101-restricted naturally processed immunodominant peptide identified in a recent-onset type 1 diabetic patient (56).

Binding to DR4 variants. The DR4 subtypes were particularly efficient in binding GAD-derived peptides. As mentioned above, some peptides, such as G176–195, G266–285, G511–525, and G556–575 bound to the majority of the DR4 variants with a high affinity (IC_{50} ranging between 3 and 50 µmol/l). This apparent DR4 promiscuity was not due to binding to the DRB4*0101-encoded molecule common to all DR4 haplotypes analyzed because similar results were observed with DR4-BLCLs expressing both DRB1 and DRB4 genes and with transfectants expressing only the DRB1*04-encoded molecules. DRB4*0101 transfectants, however, also bound G556–575, albeit with a low affinity (IC_{50} 135 µmol/l). For this peptide, it cannot be excluded that assay results obtained with $DR4^+$ BLCLs reflected competition of the peptide for binding to both DR-encoded molecules.

Among the four peptides that showed DR4 promiscuity, three have been shown to be recognized by T-cells from DRB1*0401 transgenic mice immunized with recombinant human GAD protein (57,58). Furthermore, G266–285 and G556–575 also correspond to natural T-cell epitopes identified in a recent-onset DRB1*0401-positive type 1 diabetic patient (59). By contrast, the G176–195 peptide, which bound to all DR4 variants with very high affinity, has not been identified as antigenic in DR4-transgenic mice or type 1 diabetic patients.

Some peptides showed DR4 subtype-specific interactions: G66–85 bound specifically to DRB1*0403 and G486–505 to DRB1*0405. Other peptides were specific for a group of DR4 protective subtypes (G191–205 and G336–355 bound to DRB1*0403 and 0406), whereas G246–265 was only found associated to the predisposing DRB1*0401, 0402, and 0405 alleles.

Binding of IA-2-derived peptides to DR and DQ molecules. Conclusions drawn from data with intracellular IA-2 (IA-2ic) peptides were overall similar to those obtained with GAD65 peptides. Several IA-2ic peptides bound with affinity to DR and/or DQ molecules. All DR and DQ alleles were able to bind more than one IA-2ic peptide with high or intermediate affinity (IC_{50} <100 µmol/l), with the exception of the DRB4-encoded molecule which did not bind any IA-2ic peptide. Binding efficiencies of DR and DQ alleles, and of type 1 diabetes predisposing and protective alleles were similar. These results are shown in Fig. 3.

Allele-specific binding was observed in a few cases: I641–660 and I661–680 binding to DQB1*0301, I601–620 and I681–800 binding to DQB1*0302, and I771–790 binding to DRB1*0401. The majority of peptides, however, either bound to a limited number of alleles (I821–840 bound to DQB1*0301, 0302, and 0602; I941–960 bound to DRB1*0403 and 0406) or bound promiscuously to a large panel of DR and DQ alleles. Such promiscuous binding was striking for three COOH-terminal peptides (I921–940, I951–970, and I961–980) that bound to most alleles studied.

Peptide binding motifs. The amino acid sequence of peptides usually contained anchor residues matching the known peptide-binding motif of the HLA class II allele to which they were bound. However, some peptides with an apparent adequate binding motif sequence did not bind to the corresponding allele, whereas others bound efficiently although they had a suboptimal sequence.

Using the DQB1*0602 peptide-binding motif, Ettinger and Kwok (29) recently tested in a competition assay on affinity-purified DQ molecules a set of nine GAD65 peptides matching the binding motif and found efficient binding for five of them (IC_{50} <70 µmol/l). For three of these peptides, our results were concordant with the previous study: G334–344, G396–406, and G503–513 bound to the DQB1*0602 molecule with high affinity. The two other peptides (G91–101 and G116–126) did not bind detectably in our assay. However, we identified four additional peptides that bound efficiently to DQB1*0602, although they did not match the peptide-binding motif (G41–55, G336–355, G401–415, and G411–425). Thus, the efficient binding of a peptide to a given allele does not always correlate with the presence or absence of the motif within the sequence of the peptide, as previously reported (13,14).

Binding of promiscuous peptides may be due to the presence of consensus anchor registers for multiple HLA class II alleles, at least in some cases. Alternatively, the presence of overlapping peptide-binding motifs for different HLA class II molecules could explain promiscuous binding patterns.

DISCUSSION

Understanding of the nature of peptide-HLA class II interactions has been greatly advanced in the last years by structural studies including crystallography of DR molecules, sequencing of class II–eluted naturally processed peptides, and measurement of peptide binding to DR and DQ molecules using large libraries of unrelated peptides and truncated or substituted analogs of known binders. Because crystal structure of HLA-DQ molecules is as yet unavailable, analysis of these molecules has been based on the assumption that the mode of peptide binding to the two isotypes should be fairly homologous. Consensus sequence motifs required for peptide binding to HLA class II molecules have now been described for many class II alleles. Such peptide-binding motifs are useful for identifying potential immunodominant epitopes within autoantigenic proteins. However, it has been shown that the presence or absence of a motif does not always correlate with binding to the corresponding allele. This inability of the binding motif to correctly predict all peptides that will bind to the corresponding allele indicates that there are other factors involved in determining the motif, such as the overall amino acid composition of the peptide, which may affect the ability of the peptide to interact with the peptide-binding groove (60,61).

We have developed a cellular binding assay for studying specific peptide-HLA class II interactions for a large number of DR and DQ alleles in which test peptides are used to compete against binding of a biotinylated reporter peptide. When cells in exponential growth are used, the assay is highly reproducible. Because preparation of fixed homozygous cells is significantly easier, cheaper, and more rapid than purification of HLA class II molecules, our assay should facilitate screening of (auto-)antigens for candidate epitope peptides. In addition, it is possible that the conformation of class II mol-

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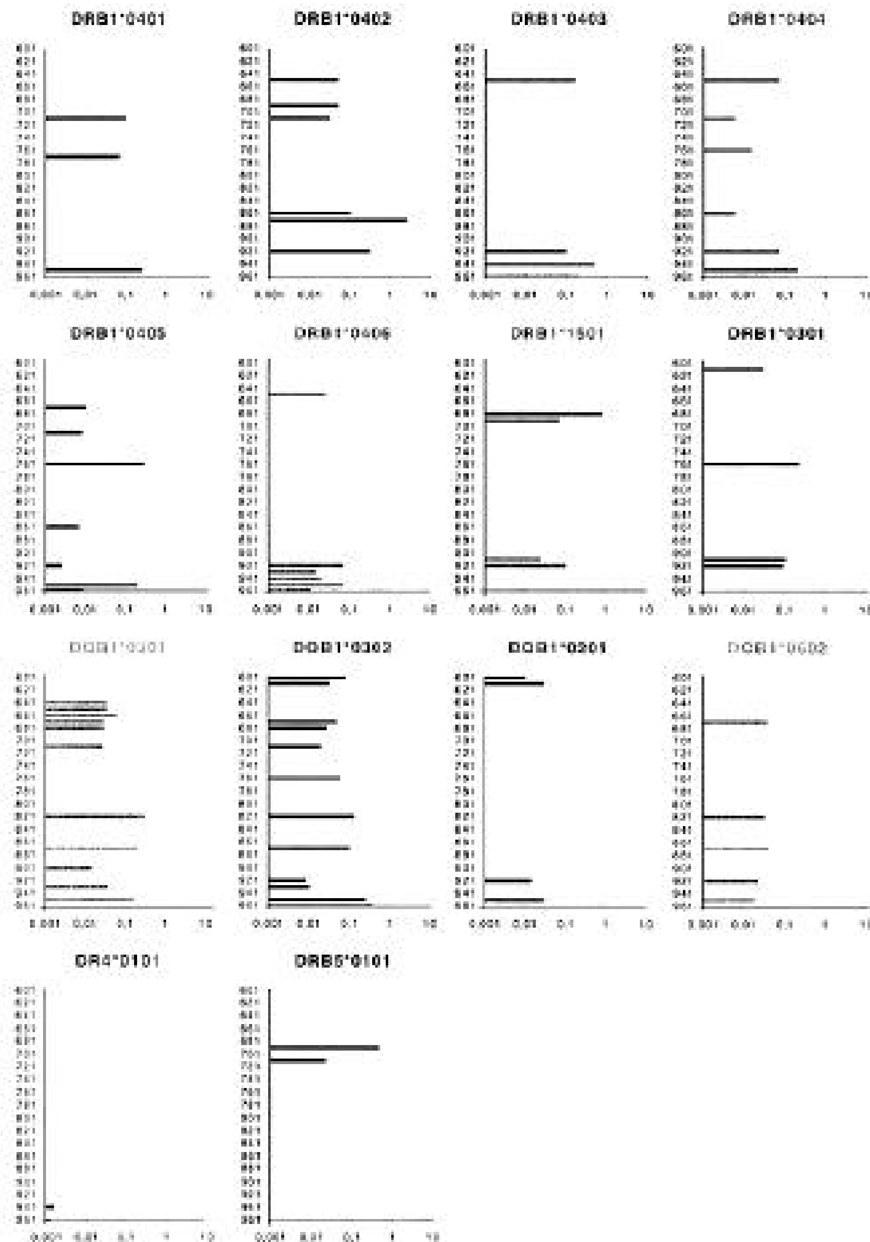

FIG. 3. Binding of overlapping peptides spanning the intracytoplasmic part of IA-2 to DR and DQ alleles. Biotinylated peptide was incubated with cells in the absence or presence of various concentrations of 20mer IA-2 competitor peptides.

ecules purified in the presence of detergent is modified compared with that of intact cells. Because reporter peptide concentrations in the cellular assay (1–3 µmol/l) are at least 1–2 logs higher than those used in assay with purified class II molecules (53), maximal molar excesses of competitor peptide that can be used in our assay are lower than in the alternative assay, so that detection of competition is limited to peptides with high or intermediate affinity (<100 µmol/l). However, for such peptides, the cellular assay allows highly sensitive measurement of variations in peptide affinity, as demonstrated in our recent analysis of the effect of single substitutions on binding affinity of two GAD65 epitopes (E.H.-H., P.M.v.E., unpublished observations).

Using the novel binding assay, we have tested a set of overlapping peptides spanning most of the sequence of two major autoantigens in type 1 diabetes, GAD65 and IA-2, both of which are the targets of B- and T-cell responses. Binding experiments were performed on the majority of type 1 diabetes–predisposing or –protective DR and DQ alleles, by contrast to previous studies that focussed on a few DR or DQ alleles (29,57,54). Our data provide a binding inventory for GAD and IA-2 peptides that can be useful for mapping natural epitopes and predicting peptide-specific responses induced by preventive immunization. Furthermore, our data may allow further insights into the mechanisms of HLA–type 1 diabetes associations.

First, both predisposing and protective class II molecules were able to bind multiple peptides derived from the two autoantigens with high affinity, an observation incompatible with the hypothesis that type 1 diabetes results from the exclusive capacity of type 1 diabetes–predisposing alleles to present autoantigenic epitopes to T-cells. Second, although a large variety of peptides bound to both predisposing and protective molecules, some of them, such as peptides derived from the NH_2-terminal part of the GAD65 protein, only bound to one or a group of HLA class II protective molecules. Interestingly, the majority of proliferative T-cell responses to GAD65 in recent-onset type 1 diabetic patients has been con-

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fined to the COOH-terminal as well as the central region of the GAD protein (46,47,62). It is not determined whether peptides that preferentially bind to type 1 diabetes–protective molecules can induce negative deletion of autoreactive T-cells as reported for experimental autoimmune encephalo-myelitis (63), positively select regulatory T-cells within the thymus, or preferentially activate peripheral T-cells of the Th2 phenotype. Furthermore, if such peptides were the more abundant peptides naturally generated for presentation, then type 1 diabetes–predisposing molecules would not be capable of efficiently presenting these peptides and this might impair thymic deletion processes.

Interestingly, the DQB1*0602 allele has also been reported to bind very efficiently certain insulin peptides, an autoantigen that was not investigated in the present study (29). In addition, recent data indicate that DQB1*0602 is the most SDS stable of several DQ alleles studied (64), suggesting that this molecular property of DQ0602 plays a role in type 1 diabetes protection. Because peptide loading is associated with SDS stability of MHC class II dimers, the increased stability of DQB1*0602 could result in determinant capture of diabetogenic peptides, outcompeting susceptible alleles for peptide binding, or could result in deletion of diabetogenic T-cells in the thymus.

Some GAD65 and IA-2ic–derived peptides bound in a promiscuous fashion to the majority of alleles studied. For instance, G246–265, homologous to a portion of the P2-C protein of Coxsackie B4 virus, bound to a variety of alleles, at variance with the high degree of allelic specificity initially proposed by Kwok et al. (22) on the basis of binding experiments with DQ molecules. In addition to DQB1*0302, we found this peptide to bind very efficiently to several DR4 subtypes as well as to the DRB5*0101 molecule. Interestingly, peptide G248–257 has been identified as a naturally processed T-cell epitope presented by the DRB5*0101 molecule in a recently diagnosed type 1 diabetic patient expressing the DRB1*1302–1501 phenotype (56). In addition to G246–265, other promiscuous peptides were present within the COOH-terminal GAD65 and IA-2 regions. The rules governing binding of such promiscuous peptides are not fully understood. Within their sequence, it was sometimes possible to identify allele-specific anchor residues corresponding to the preference of each bound class II allele, with a partial or even complete overlap of registers. This was frequently the case for peptides binding to DRB1*1501 and DRB5*0101, which contained anchor motifs for both molecules with a frame shift of three amino acids, as previously described for an immunodominant epitope of the myelin basic protein (MBP) 87–99 (19,51).

However, binding of promiscuous peptides may also be explained by the fact that they possess consensus recognition elements, i.e., "supertype-like sequences," that allow binding to multiple alleles. Recent data show that a set of at least seven different DR alleles, including DRB1*0401, DRB1*1501, and DRB5*0101, select overlapping repertoires of peptide ligands so that binding to HLA-DR molecules in a strongly degenerate fashion is a relatively common occurrence (64,65). Nearly all peptides binding to these DR molecules bear a motif characterized by a large aromatic or hydrophobic residue in position P1 and a small noncharged residue in position P6. Additional effects are associated with secondary anchors at positions P4, P7, and P9. This information has enabled authors to define a quantitative algorithm for predicting binding to these DR alleles. Interestingly, this set of alleles includes DRB5*0101, which, although less abundantly expressed at the cell surface than DRB1-encoded molecules, is known to behave as a functional restriction element (51,56,65,66). Indeed, we observed that DRB5*0101 peptide-binding specificity was often similar to the specificity of several other DR variants (in particular the various DR4 subtypes), so that promiscuous peptides usually bound to DRB1*0401–0406, DRB1*1501, and DRB5*0101. By contrast, none of the GAD65 promiscuous peptides (with the exception of G511–525) bound to DRB1*0301, which bound a set of peptides largely distinct from other alleles, a fact also recently observed by Geluk et al. (54). This fits well with data of Southwood et al. (65) showing that at least two additional groups of alleles exist (64). The first group comprises molecules (DRB1*0405, 0802, and 1101) whose selectivity possesses significant overlap with the DR supertype. By contrast, the second group of alleles (DRB1*1201 and 0301) selects a repertoire with little overlap with the DR supertype. This observation may provide a basis for the known synergistic predisposing effect of the DR3 and DR4 haplotypes that bind clearly distinct sets of peptides.

An additional explanation to binding of promiscuous peptides to multiple class II molecules is that certain peptides may bind outside of the binding groove in the superantigen staphylococcal enterotoxin B (SEB) binding site. This has previously been shown for the B(10–30) peptide from the B-chain of insulin, which almost entirely contains the B(9–23) peptide associated with type 1 diabetes (67).

Our data must also be considered in view of the known association between HLA class II polymorphism and the prevalence of GAD65 or IA-2 antibodies in type 1 diabetes. Indeed, the presence of the DR3 and DQ2 molecules, which bind very few GAD65 peptides but several IA-2 peptides, is positively associated with a high prevalence of anti-GAD antibodies but negatively associated with the presence of IA-2 antibodies (5,68,69). This inverse relationship between the capacity of DR3 to present autoantigenic peptides to T-cells and its capacity to induce humoral immunity to GAD65 may indicate a shift towards an antibody-dominated response to autoantigens in the presence of the DR3-DQ2 haplotype (45,70).

Finally, our data provide some elements for understanding the mechanisms of dominant protection conferred by the DR15-DQ6 haplotype. In DR15/DR3 or DR15/DR4 heterozygous individuals, antigen-presenting cells bear both susceptible and protective alleles on their surface, so that there may be competition for binding of diabetogenic peptides derived from the same antigenic region in a phenomenon of determinant capture (34,35). Competition for binding of autoantigenic peptides may especially concern protein fragments binding to numerous HLA class II molecules such as the COOH-terminal regions of GAD65 and IA-2ic and the central part of GAD65; the interest of these regions is enhanced further by previous reports that immunodominant epitopes presented by associated as well as protective HLA class II haplotypes are located in these regions (55–58). Peptides containing two overlapping or superimposed binding motifs for two HLA-DR molecules expressed on the same haplotype (for instance, DRB1*1501 and DRB5*0101) are excellent candidates for immunodominant epitopes whose preferential

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presentation by protective alleles may render them unavailable for presentation by predisposing alleles. As an additional mechanism for HLA-mediated protection from type 1 diabetes, peptide presentation by protective alleles may either be unable to activate diabetogenic T-cells or deliver a tolerogenic or anergizing signal.

In conclusion, we have established an inventory that is likely to include most GAD65 and IA-2ic peptides with intermediate or high binding affinity for HLA class II alleles predisposing to or protecting from type 1 diabetes. It remains to be determined which of these peptides can be naturally processed for efficient presentation to CD4 T-cells. We show that type 1 diabetes predisposing and protective alleles are indistinguishable with respect to the number and affinities of autoantigenic peptides they bind. Moreover, we identify three regions in autoantigenic proteins that contain multiple immunodominant epitopes and are therefore especially likely to be involved in phenomena of competition for autoantigen presentation. Although the determination of peptide-binding affinities does not provide direct information on their antigenicity, it can be tremendously useful in the localization of key T-cell epitopes of GAD65 and IA-2 proteins that initiate type 1 diabetes. Good-affinity binding peptides can then be tested directly for their functional role as T-cell epitopes. Indeed, we usually observed that GAD65 T-cell epitopes identified in humans or in transgenic mice (56–59) were high affinity binders in our assay. In other HLA-associated disease models, immunodominant T-cell epitopes of self-peptides have been found to bind with high affinity to the HLA class II predisposing molecules, and the T-cell data have usually been consistent with the peptide-binding experiments (51,71).

The peptide inventory established in this study may also be useful in another respect. Immunization with synthetic peptides derived from a protein antigen can elicit a different spectrum of responses from that obtained by immunization with the whole protein, indicating that the T-cell repertoire includes clones responsive to cryptic epitopes. Our binding inventory is likely to include peptides representing subdominant/cryptic epitopes that may be useful for deviating β-cell–specific T-cell response in genetically type 1 diabetes–susceptible individuals.

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