Identification of Naturally Processed HLA-A2–Restricted Proinsulin Epitopes by Reverse Immunology

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Type 1 diabetes is thought to result from the destruction of β-cells by autoantigen-specific T-cells. Observations in the NOD mouse model suggest that CD8$^+$ cytotoxic T-cells play an essential role in both the initial triggering of insulitis and its destructive phase. However, little is known about the epitopes derived from human β-cell autoantigens and presented by HLA class I molecules. We used a novel reverse immunology approach to identify HLA-A2–restricted, naturally processed epitopes derived from proinsulin, an autoantigen likely to play an important role in the pathogenesis of type 1 diabetes. Recombinant human proinsulin was digested with purified proteasome complexes to establish an inventory of potential COOH-terminals of HLA class I–presented epitopes. Cleavage data were then combined with epitope predictions based on the SYFPEITHI and BIMAS algorithms to select 10 candidate epitopes; 7 of these, including 3 with a sequence identical to murine proinsulin, were immunogenic in HLA-A2 transgenic mice. Moreover, six of six tested peptides were processed and presented by proinsulin-expressing cells. These results demonstrate the power of reverse immunology approaches. Moreover, the novel epitopes may be of significant interest in monitoring autoreactive T-cells in type 1 diabetes. *Diabetes* 54:2053–2059, 2005

Type 1 diabetes is thought to result from the destruction of β-cells by T-cells recognizing β-cell–expressed peptides in the context of HLA molecules (1). Although the association of major histocompatibility complex (MHC) class II polymorphism with genetic type 1 diabetes susceptibility (2) has stimulated more intense interest in the role of CD4$^+$ T-cells in the disease, observations made in the NOD mouse model document an essential role of CD8$^+$ T-cells (3). CD8$^+$ cells are abundant in islet infiltrates (4,5) and are required for the progression to diabetes after the transfer of bulk NOD T-cells to irradiated recipients (6,7). Some CD8$^+$ clones from diabetic mice can transfer the disease to lymphocyte-deficient recipients (8,9). Moreover, the absence of insulitis in mice lacking β_2-microglobulin or injected with anti-CD8 antibodies suggests that CD8$^+$ T-cells may mediate the initial insult to β-cells (10–12).

Identification of the β-cell antigens and antigenic epitopes recognized by CD8$^+$ T-cells is therefore an important objective of research aiming to understand, monitor, and prevent type 1 diabetes (13). Data obtained in the NOD mouse suggest that proinsulin, the only type 1 diabetes autoantigen with a β-cell–restricted expression, may play an important role in the disease pathogenesis (14). The effective induction of insulin tolerance (15) and the genetic deletion of one murine proinsulin isoform (16) protect NOD mice from diabetes, whereas similar manipulations of other autoantigens do not affect type 1 diabetes onset (17,18). Conversely, deletion of the other, tolerizing murine isoform of proinsulin accelerates diabetes (19). Moreover, insulin is recognized by diabetogenic murine T-cells, and murine CD8$^+$ T-cells recognizing the insulin epitope B15-B23 can be isolated from islet-infiltrating lymphocytes at an early stage of disease (20–22). In humans, the importance of tolerance to proinsulin is underlined by the fact that the level of proinsulin expression in the thymus shows a negative correlation with the risk of developing type 1 diabetes (23). Strongly Th1-biased T-cell responses against proinsulin provide further evidence for its role as an important autoantigen in patients with type 1 diabetes (24).

In view of these observations, it is important to study T-cell responses to HLA class I–restricted proinsulin epitopes in humans and humanized mouse systems. Although HLA-DR–restricted proinsulin epitopes have been identified (24), HLA class I–restricted epitopes have not been described. Reverse immunology is a strategy facilitating the identification of HLA class I–presented epitopes (25). In its most common version, it entails screening antigen sequences using algorithms capable of predicting peptides binding to a given class I allomorph. In several algorithms, exemplified by BIMAS (26), predictions are based exclusively on the predicted affinity of the peptide/HLA interaction. Others, such as SYFPEITHI (27), also predict class I binding but are

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CTL, cytotoxic T-cell; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing.

largely trained with natural epitope data; this is expected to increase the likelihood that a given peptide is a naturally processed epitope.

It is typical to find that approximately two of every three peptides predicted on the basis of class I binding affinity are not naturally processed epitopes (false positives) (28). True epitopes with low class I binding affinities also tend to be missed by algorithms based on class I binding (false negatives). In most cases, such prediction failures are due to peptide filtering in intracellular antigen processing. Consequently, it may be possible to reduce failure rates by experimentally simulating the major processing steps and combining the results with predictions based on class I binding affinity. The major antigen-processing steps are degradation by cytoplasmic proteasome complexes and peptide transport from the cytoplasm to the endoplasmic reticulum by transporters associated with antigen processing (TAPs) (29). Proteasome complexes are presently considered to be responsible for essentially all cleavages at the COOH-terminal end of class I ligands, whereas NH_2-terminal ends are thought to be produced frequently by trimming peptidases in the cytoplasm or endoplasmic reticulum (30). Therefore, the presence of an experimentally determined proteasome cleavage site after the COOH-terminal of a peptide can be used as a predictor of epitope status. Indeed, application of this strategy allowed Kessler et al. (31) to identify epitopes derived from the tumor antigen PRAME with a lower failure rate. Given clear statistical and experimental evidence that TAP transport acts as an epitope filter (32,33), determining the efficiency of TAP transport should also ameliorate epitope prediction. However, application of a TAP filter is complicated by the fact that TAP selection acts mainly on epitope precursor peptides with NH_2-terminal extensions, which will rarely be known for a given epitope (32).

In this study, we used a reverse immunology approach with experimental proteasome digestions to identify HLA-A2–restricted epitopes derived from the type 1 diabetes autoantigen proinsulin. We identify several novel epitopes that are naturally processed by proinsulin-expressing cells. These findings may be useful for developing strategies for monitoring autoimmune $CD8^+$ T-cells in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

Cells and reagents. The lymphoblastoid cell lines 721 and 721.174 have been previously described (34). Line 721 expresses mainly immunoproteasome complexes, whereas line 721.174 harbors exclusively housekeeping proteasome complexes; similar cleavage profiles have been observed for the two proteasome types (not shown). For kill assays with mouse cytotoxic T-cells (CTLs), transfectants expressing the HLA-A2 single-chain molecule HHD (see below under "Generation and use of murine cytotoxic T-cell lines") were used; TAP-deficient RMA-S/HHD transfectants were used for peptide pulsing, and HeLa/HHD transfectants were used for vaccinia infections. Transfectants were produced by transfection of a previously described genomic HHD construct (35) conferring G418 resistance. Lyophilized recombinant proinsulin was kindly provided by Eli Lilly (Indianapolis, IN); purity was confirmed in silver-stained SDS-PAGE gels and by reversed-phase chromatography. Peptides were purchased from NeoMPS (Strasbourg, France) and were at least 80% pure.

Proteasome digestions. Proteasome complexes were purified by immunoaffinity chromatography from polyethylene-glycol (PEG 6000; Fluka, Buchs, Switzerland) precipitated cytosol fractions, prepared as previously described (36) using monoclonal antibody MCP21 (37) coupled to Sepharose 4B beads (Amersham Biosciences, Orsay, France). Proteasome was eluted using 100 mmol/l triethanolamine (pH 11.5), concentrated and dialyzed against the digestion buffer we previously used (36). Fractions were monitored for proteasome content using the fluorogenic substrate Suc-LLVY-amido-methylcoumarin (Bachem, Weil am Rhein, Germany). AAF-chloro-methylketone (Bachem), an inhibitor of tripeptidyl-peptidase II, had no effect on Suc-LLVY-amido-methylcoumarin hydrolysis by immunoaffinity-purified proteasome. The complete absence of tripeptidyl-peptidase II contamination was also confirmed using Western blots with a specific antibody.

Before proinsulin was digested, cysteines were carboxymethylated by incubation with 10 mmol/l dithiothreitol for 60 min then incubated with 20 mmol/l iodoacetic acid for 20 min. Carboxymethylated proinsulin (40 μg) was incubated at 37°C for various periods with 2 μg purified proteasome in 20 mmol/l HEPES buffer (pH 7.3) containing 1 mmol/l ethylene-bis(oxyethylenenitrilo)tetraacetic acid (Fluka), 5 mmol/l $MgCl_2$, and 0.5 mmol/l 2-mercaptoethanol. Digestions were stopped by adding 8% acetonitrile and fractionated by reversed-phase chromatography (μRPC C2/C18 column; Amersham) using 0.1% trifluoroacetic acid (eluent A), 80% acetonitrile/0.08% trifluoroacetic acid (eluent B), and a gradient of 2–90% eluent B over 90 min.

To identify digestion products, fractions were analyzed by matrix-associated laser desorption ionization/time of flight mass spectrometry with a Voyager STR spectrometer coupled with delayed extraction (Applied Biosystems, Warrington, U.K.). Mass accuracy was 20–40 ppm. Sequences were confirmed by tandem mass spectrometry analysis on a Micromass Q-TOF instrument (Waters, Elstree, U.K.).

Prediction and determination of HLA-A2 and TAP binding affinities. HLA-A2 binding affinities were predicted using both the SYFPEITHI (available from http://www.syfpeithi.de) and BIMAS (available from http://bimas.cit.nih.gov) algorithms. HLA-A2 and TAP binding affinities were determined experimentally in previously described competitive binding assays (38,39).

Production of a recombinant vaccinia virus expressing preproinsulin. The human preproinsulin coding sequence was amplified from human pancreas cDNA (PCR-ready cDNA; Ambion, Huntington, U.K.) using a high-fidelity thermostable DNA polymerase (Advantage HF kit; Invitrogen, Cergy Pontoise, France) and the primers 5′ TTAGATCTACCATGGCCCTGTG GATGC (sense) and 5′ AAGGTACCTACTAGTTGCAGTAGTTCTCCA (antisense). The PCR product was cloned in pCR Blunt (Invitrogen), sequenced to confirm the absence of errors, and subcloned into the vaccinia virus transfer vector pSC65 (40). The resulting plasmid and purified wild-type vaccinia DNA were co-transfected into CV-1 cells that were simultaneously infected with strain WR wild-type vaccinia virus (ATCC, Manassas, VA). Recombinant viruses were selected in plaque assays with blue/white selection and amplified in HeLaS3 cells, following previously described standard protocols (40). A control virus expressing the intracellular portion of the type 1 diabetes autoantigen IA-2 (41) was produced in the same fashion. The expression of preproinsulin was confirmed by Western blot analysis; 200,000 HeLaS3 cells infected with the vaccinia virus at a multiplicity of infection of 10 were lysed in sample buffer; proteins were then separated in a 15% SDS-polyacrylamide gel, blotted on a polyvinylidine fluoride membrane, and stained with rabbit antibodies to proinsulin used at 200 ng/ml (H-86; Santa Cruz Biotechnologies, Heidelberg, Germany) using an enhanced chemiluminescence detection system (Supersignal West Pico; Perbio Science, Brebières, France).

Generation and use of murine cytotoxic T-cell lines. CTL lines were generated by immunization of HHD2 transgenic mice (35). HHD2 mice were immunized by subcutaneous injection at the base of the tail of 100 μg of A2-restricted peptides together with 140 μg of the MHC class II–restricted helper peptide T13L (TPPAYRPPNAPIL) emulsified in incomplete Freund's adjuvant (Difco, Detroit, MI). For each HLA-A2 ligand studied, five mice were injected. Then 12 days after injection, spleens were removed and splenocytes were restimulated using irradiated (3,500 rad) HHD2 lymphoblasts previously pulsed for 2 h with 10 μg/ml of peptide at a concentration of 5×10^6 cells/ml. Restimulated cells were tested 6 days later in chromium release assays using HHD-transfected HeLaS3 or TAP-deficient RMA-S cells pulsed with proinsulin peptides or a control HLA-A2 ligand (GILGFVFTL). CTL lines showing specific target cell lysis were cultured in RPMI with 10% FCS, 10% T-cell growth factor, and 0.1 mmol/l 2-mercaptoethanol and maintained by weekly restimulation with peptide-pulsed HHD2 lymphoblasts. T-cell lines were also tested for recognition of HeLa target cells synthesizing preproinsulin. Typically, 10^6 target cells were infected with recombinant or wild-type vaccinia virus for 2 h (unless otherwise indicated) at a multiplicity of infection of 10 in medium without serum. Target cells were then labeled for 1 h at 37° with 100 μCi of ^{51}Cr, washed four times, and incubated in aliquots of 5×10^3 cells/well for 4 h in V-bottom 96-well plates with killer cells. Results are presented as the means of triplicates calculated as follows: 100 × (experimental release − spontaneous release)/(total release − spontaneous release).

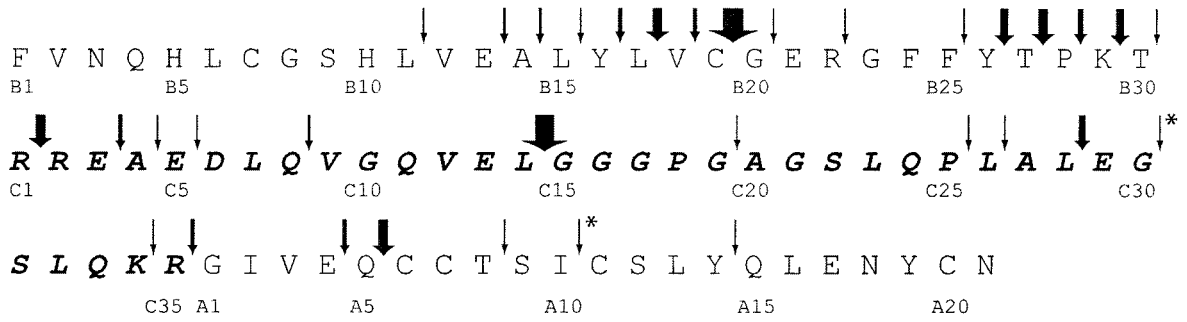

FIG. 1. Proteasome cleavage sites in proinsulin. Data are pooled from three digests of carboxymethylated proinsulin by 20S proteasome complexes purified from 721 B (mainly immunoproteasome, two digests) or 721.174 (housekeeping proteasome) cells. Both cleavages before the NH_2-terminal and after the COOH-terminal of peptides identified by mass spectrometry are considered. The number of cleavage events after each residue is represented by the thickness of the arrow. *Cleavages observed in additional digests of reduced unmethylated proinsulin.

RESULTS

To identify HLA-A2–restricted proinsulin epitopes, we began by digesting proinsulin with purified proteasome complexes, reasoning that most or all HLA class I–presented epitopes should be followed by a proteasome cleavage site (29). The 20S proteasome complexes were purified from human B-cell lines expressing immuno- or constitutive proteasome complexes (37). Recombinant proinsulin was reduced and carboxymethylated to eliminate disulfide bridges and digested with purified proteasome.

A summary of three digestions of carboxymethylated proinsulin is shown in Fig. 1. Overall, the frequency of cleavages was highest in the B chain where two clusters of cleavage sites were found (B13–B20 and B25–C1); an isolated B chain fragment has previously been found to be an efficiently cleaved proteasome substrate (42). In contrast, cleavage sites were less frequent in the C-peptide and A chain, which contain a lower number of residues preferred by the proteasome. Some cleavage sites were clearly preferred (e.g., B19, C14), whereas many were used only once.

In the next step, we sought to identify potential HLA-A2 ligands in which the COOH-terminal anchor residue was used as a proteasome cleavage site. To this end, we applied the BIMAS and SYFPEITHI algorithms to the proinsulin sequence and established, for each algorithm, separate lists of the 9- and 10-mer peptides (n = 10 each) with the highest binding scores for HLA-A2 (26,27). Because only 40% each of the highest scoring 9- and 10-mer peptides were identical between the two algorithms (not shown), these lists contained a total of 32 peptides. Candidate A2-restricted epitopes were then selected according to the following criteria (Table 1 and Fig. 2). A first group of four peptides (two 9-mers, P2 and P4, and two 10-mers, P1 and P8) scored high in both algorithms and had proteasome-produced COOH-terminal ends. A second group of three peptides (P5, P6, and P7) also ended with proteasome cleavage sites and scored high in SYFPEITHI but not in BIMAS, thus representing candidate epitopes with putative low A2 binding affinity. Finally, to test some peptides with high predicted A2-binding scores without considering the other parameters, we selected the two peptides with the highest scores in BIMAS (P3 and P9) and a peptide with high scores in both algorithms whose COOH-terminal was not produced by the proteasome (P10). The selected 10 peptides are distributed over the entire proinsulin sequence. Note that all candidate epitopes in the C-peptide contain several substitutions relative to the murine proinsulin sequences, whereas the candidate epitopes in the human B and A chains are entirely conserved in the mouse, with the exception of peptide A1-A10, which harbors a single conservative substitution (Table 1).

TABLE 1
Candidate A2-restricted epitopes

No.	Position	Sequence (human PI)	Mouse PI-1 (PI-2)	Binding score		HLA-A2 affinity	TAP affinity	Proteasome cleavages*
				SYFPEITHI	*BIMAS*			
1	B9-B18	SHLVEALYLV	P(-)---------	19	0.4	0.8	6.7	3
2	B10-B18	HLVEALYLV	---------	27	22.3	2.5	42	3
3	B18-B27	VCGERGFFYT	---------	—	20.5	27	91	7
4	C6-C14	DLQVGQVEL	-P--E(A)-L--	25	1.6	370	495	14
5	C22-C30	SLQPLALEG	D--T----V	18	—	122	>1,000	1
6	C27-C35	ALEGSLQKR	---VAR(Q)---	19	—	154	443	3
7	C31-A5	SLQKRGIVEQ	AR(Q)------D-	20	—	185	482	7
8	A1-A10	GIVEQCCTSI	---D------	21	6.0	6.3	12	1
9	A8-A16	TSICSLYQL	------	17	0.3	1.2	4.8	0
10	A12-A20	SLYQLENYC	------	15	87.4	6	0.9	0
Reference peptide (FLPSDFFPSV/ RRYNASTEL)		—	—	24	2,310	1	1	NA

Sequence identity between human and murine proinsulin is indicated by a dash. The binding score indicates a rank ≤11 for the indicated algorithm. HLA-A2 and TAP affinities are expressed as relative affinities (i.e., IC_{50} of test peptide/IC_{50} of reporter peptide). *Number of proteasome cleavages after COOH-terminal. NA, not applicable; PI, proinsulin.

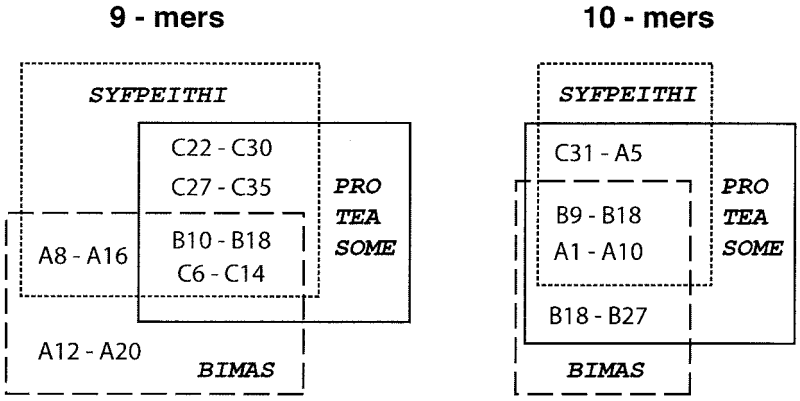

FIG. 2. Venn diagram visualizing algorithm predictions and proteasome generation for the 10 selected candidate epitopes. Inclusion in the proteasome box indicates at least a single cleavage event after the peptide COOH-terminal, as shown in Fig. 1. Inclusion in the SYFPEITHI or BIMAS boxes indicates that the peptide was among the 10 peptides with the highest scores predicted by the algorithm.

To further characterize the 10 selected peptides, we measured their HLA-A2 and TAP binding affinities in previously described competitive binding assays (38,39). In both assays, values <10 are considered to reflect high affinity, values of 10–100 are intermediate, and values >100 are low affinity. Of interest, all peptides located in the C-peptide had low HLA-A2 and TAP affinities, whereas all B or A chain peptides had high or intermediate affinities (Table 1).

To study the immunogenicity of the candidate epitopes, we used the 10 selected peptides to immunize HHD2 transgenic mice expressing a single chain HLA-A2/β_2m molecule (35). Because of the significant diversity between human and murine C-peptides but not B and A chains (Table 1), putative epitopes derived from the human C-peptide were more likely to elicit high-affinity T-cell responses in HHD2 mice.

We observed that 7 of the 10 candidate epitopes were immunogenic in HHD2 mice (Table 2). Among these, peptides C27-C35 and A12-A20 were recognized by CTL lines displaying low (<10% after second restimulation) specific lysis that were lost after two restimulations; for the other lines, a minimum of five kill assays were performed, with rates of specific lysis equal or superior to those obtained in experiment 2 (Table 2). The lack of T-cell responses may have been due to T-cell tolerance for peptides B18-B27 and A8-A16, which are completely conserved in the murine proinsulin sequence. Peptide C6-C14 had a very low HLA-A2 binding affinity, which may explain our inability to generate a CTL line against it. Of the five peptides stimulating CTL lines that could be maintained in culture and that displayed >20% specific lysis, three were derived from the B or A chains and nonconserved, with the exception of a conservative substitution in peptide A1-A10. The two others were conserved C-peptide epitopes.

TABLE 2
Immunogenicity of candidate epitopes

No.	Position	Responding mice (of 5)	Lysis (%) Experiment 1	Lysis (%) Experiment 2
1	B9-B18	2	8.5/42.1	12.3/57
2	B10-B18	2	7.2/8.6	29/25.4
3	B18-B27	0	—	—
4	C6-C14	0	—	—
5	C22-C30	2	18.5/5.0	29.2/22.7
6	C27-C35	3	7.4/17.7/7.2	7.4/2.7
7	C31-A5	1	10.6	34.7
8	A1-A10	3	45.0/27.0/30.0	40.8 /10.4/40.7
9	A8-A16	0	—	—
10	A12-A20	1	11.8	6.2

Experiment 1 corresponds to the test of the lines after the first stimulation in vitro and experiment 2 was performed after additional restimulations. Lysis <10% was considered low and lysis >20% was considered efficient. The data show lysis for CTL lines from individual responding mice, separated by slashes.

Given that proinsulin peptides are likely to be presented to T-cells undergoing thymic education (23), the avidity of T-cells recognizing conserved peptides is likely to be lower than that of nonconserved ones. To assess the avidity of T-cell recognition, we tested the lysis of target cells pulsed with different peptide concentrations (Fig. 3). Consistent with the assumption that proinsulin acts in thymic education of CD8 T-cells, T-cells recognizing the two completely conserved B chain epitopes (B10-B18 and B18-B27) required 10 μmol/l peptide for efficient lysis, whereas CTLs recognizing the nonconserved C-peptide epitopes displayed efficient killing of targets pulsed with 100- to 10-fold lower concentrations. Epitope A1-A10 was recognized as efficiently as the C-peptide epitopes. Therefore, either the substitution (Glu to Asp) in position 4 of this epitope alters peptide recognition by T-cell receptors or it is presented inefficiently during thymic education.

Immunogenicity of a peptide documents the presence of a specific T-cell repertoire and sufficient MHC binding

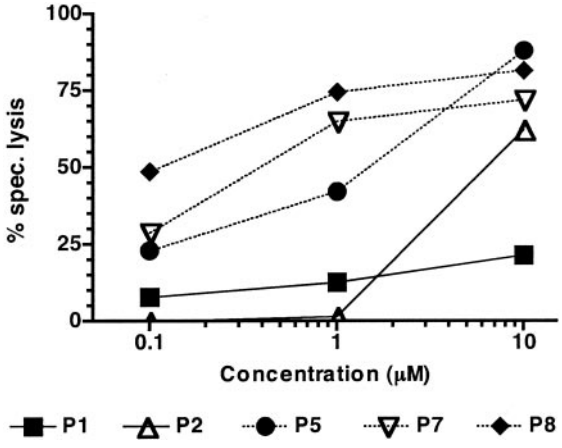

FIG. 3. Efficiency of peptide recognition by CTL lines. HHD-transfected HeLa cells were pulsed for 2 h with the indicated peptide concentrations (e.g., P1; peptide numbering refers to Table 1), washed, labeled with ^{51}Cr, and incubated for 4 h with CTLs at an effector-to-target ratio of 100. The graph shows the mean of duplicate samples. One representative of two experiments is shown. Lysis <10% was considered low and lysis >20% was considered efficient.

affinity, but is not necessarily indicative of its presentation by a cell expressing the source protein. Infection by recombinant vaccinia viruses is a frequently used means to introduce antigens into the endogenous processing pathway of antigen-presenting cells (43). We produced a recombinant vaccinia virus encoding human preproinsulin. Infection of HeLa cells by the virus resulted in a high-level expression of a protein recognized by an antibody with specificity for proinsulin (Fig. 4*A*). The molecular weight of this protein was somewhat higher than that of recombinant proinsulin, possibly because of inefficient processing of the preproinsulin signal peptide or of posttranslational proinsulin modifications.

All CTL lines that could be maintained over a longer period killed HLA-A2−expressing target cells infected with the preproinsulin-encoding vaccinia virus in a specific fashion (Fig. 4*B*); targets infected with wild-type virus or a virus encoding the type 1 diabetes autoantigen IA-2 (41) were not killed (not shown). In accordance with the results shown in Fig. 3, CTL lines recognizing the C-peptide epitopes killed targets infected for short periods and therefore were likely to present low peptide concentrations, whereas CTLs with specificity for the conserved B chain epitopes required longer infection periods for efficient killing (Fig. 4*B*). The four epitopes located in the B chain and C-peptide were efficiently presented by vaccinia-infected cells as lysis levels were equal to or higher than the lysis of cells pulsed with high peptide concentrations. However, in the case of CTLs with specificity for epitope A1-A10, recognition of vaccinia-infected targets was much less efficient than that of peptide-pulsed targets, suggesting that this epitope may be generated inefficiently by antigen-presenting cells. CTLs recognizing peptide A12-A20 also killed vaccinia-infected cells (Fig. 4*C*). Thus, for six of six epitopes that could be tested, natural peptide processing by cells expressing preproinsulin could be demonstrated.

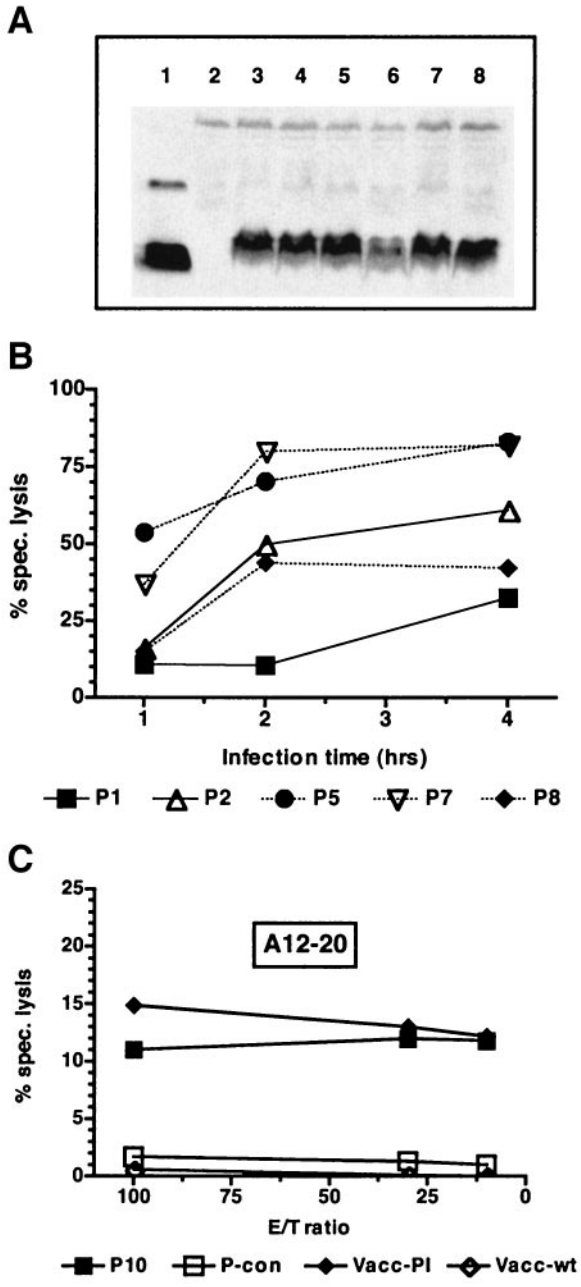

FIG. 4. Natural processing of proinsulin epitopes. *A*: HeLaS3 cells were infected with different isolates of a recombinant vaccinia virus encoding human preproinsulin (*lanes 3–8*) or a virus encoding IA-2 (*lane 2*) and tested for proinsulin expression by Western blot analysis using an antiserum against insulin. *Lane 1* contains 1 μg of recombinant proinsulin. *B*: HHD-transfected HeLa cells were infected with vaccinia/preproinsulin for the indicated periods, labeled with ^{51}Cr, and incubated with CTLs at an effector-to-target ratio of 100. Lysis of cells infected with wild-type vaccinia was <2% for all CTL lines. Shown is one of three similar experiments. *C*: Specific killing of HeLa-HHD cells pulsed with 1 μmol/l peptide A12-20 (P10) or a control peptide (P-con; GILGFVFTL) or infected with vaccinia proinsulin (Vacc-PI) or wild-type vaccinia (Vacc-wt) at different effector-to-target ratios. Note the different scale of this panel relative to panel *B*.

DISCUSSION

In this study, we used a reverse immunology approach to identify naturally processed, HLA-A2−restricted epitopes derived from the type 1 diabetes autoantigen proinsulin. This approach proved highly successful in predicting immunogenicity and natural cellular processing of the epitopes. Although at least 5 of 10 epitopes were self-peptides in the mouse model used, only 3 were nonimmunogenic. Even more impressive, six of six epitopes that could be tested were naturally processed in proinsulin-expressing cells.

Although the identified epitopes clearly have a potential for studies of proinsulin responses in diabetic and healthy humans, it is important to emphasize that neither peptide recognition by murine T-cells nor its absence indicate necessarily similar recognition profiles in humans. Species-specific differences in the T-cell repertoire, in the availability and specificity of helper T-cells, and in proteases and accessory proteins involved in antigen processing may all affect autoimmune CD8$^+$ responses. However, the identified epitopes are a reasonable starting point for studies with human T-cells (e.g., using ELISPOT assays combined with tetramer enrichment to determine the frequency, phenotype, and relation of specific cells with type 1 diabetes).

Because of the sequence differences between murine and human proinsulin, immunogenicity data obtained in HHD2 mice have the potential to reflect the situation in humans only for the epitopes that are self-peptides in both species (B9-B18, B10-B18, A12-A20, and possibly A1-A10).

Among these, the former two, which differ only by an NH_2-terminal extension in B10-B18, may be recognized by overlapping sets of T-cells. The conserved peptides were recognized by murine T-cells with low avidity, suggesting that they are presented to T-cells during thymic education.

Of the four candidate epitopes derived from the C-peptide, two were recognized with high efficiency (C22-C30 and C31-A5), one was poorly recognized (C27-C35), and one was not recognized at all (C6-C14), presumably because of its very low HLA-A2 binding affinity. If epitopes C22-C30 and C31-A5 are presented during thymic T-cell education in humans, their T-cell recognition in humans may be less efficient than in the humanized mice. However, the phenomenon of avidity maturation during disease progression, previously described in the NOD mouse (22), may also apply to human $CD8^+$ T-cells recognizing proinsulin peptides.

To our knowledge, a reverse immunology approach in which proteasome digestion data are used as a starting point has not been used before. Kessler et al. (31) used proteasome digestions of synthetic 25-mer peptides to identify peptides likely to be naturally processed among a set of algorithm-predicted peptides derived from the large tumor antigen PRAME. The small size of the proinsulin protein made it possible to invert the order of the first two screening steps and start by digesting the complete source protein; it is uncertain whether this approach can be applied to larger proteins. Our decision to use proteasome digestions as an initial screening filter was based on the concept that this protease generates all COOH-terminals of class I–presented epitopes. However, although proteasome cleavage correlates well overall with epitope status (see below), there was no quantitative correlation between proteasome cleavage after the COOH-terminal and efficiency of presentation (see Table 1). It should also be noted that epitope A12-A20, a poorly presented but naturally processed epitope, seemed to be not produced at all in proteasome digestions. This may point to limitations of digestions by 20S core proteasome complexes performed in this study; partly different cleavage products might be produced by 26S proteasome complexes or in the presence of the 11S proteasome activator. Moreover, it cannot be ruled out that cysteine carboxymethylation, required for proinsulin digestion, has some effect on proteasome cleavage.

Our data do not allow for a direct comparison of the predictive performance of the SYFPEITHI and BIMAS algorithms and of the various experimentally measured processing parameters because, in the interest of a high predictive hit rate, we selected mainly peptides scoring high in several algorithms and parameters. However, some tentative conclusions can be drawn. Proteasome cleavage after the epitope COOH-terminal was a good parameter, given that six of seven epitopes ended with a proteasome cleavage site. Excluding peptides that may be nonimmunogenic because of tolerance (B18-B27 and A8-A16), the following statements can be made about the two algorithms. SYFPEITHI predictions performed well, as seven of eight peptides with high SYFPEITHI scores were indeed epitopes (of which six could be confirmed to be naturally processed). However, BIMAS predictions were much less informative, as three peptides with very low BIMAS scores were epitopes, whereas one of the five peptides with a higher score was not. This underlines the fact that high class I binding affinity is not necessarily required for epitope status (28). In conclusion, when cytoplasmic processing is taken into account, either by actual proteasome digestion of source antigens or by using the epitope-trained SYFPEITHI algorithm, predictions are likely to be more efficient than when based merely on predictions of class I binding affinity such as in BIMAS.

Measured HLA-A2 binding affinities showed a limited correlation with epitope status. Thus, although the very low A2 binding affinity of peptide C6-C14 may explain its poor immunogenicity, the low (though slightly higher) affinity of C31-A5 and C22-C30 did not prevent immunogenicity and efficient presentation. The TAP affinities of the minimal epitopes measured did not seem to contribute to the efficiency of epitope presentation, as both of the C-peptide epitopes presented with high efficiency by vaccinia-infected cells (C22-C30 and C31-A5) had low to very low affinity. This was presumably due to the fact that, according to statistical evidence, many HLA class I–presented epitopes enter the endoplasmic reticulum as NH_2-terminal extended precursors (32). Because the three NH_2-terminal residues of a peptide have a strong influence on its TAP affinity (39) and can be removed after transport by trimming peptidases in the endoplasmic reticulum (30), low TAP affinities of minimal class I–presented epitopes do not preclude efficient presentation.

In conclusion, we have identified several HLA-A2–presented epitopes derived from human proinsulin by intracellular processing. Additional A2-restricted epitopes may be present within the proinsulin signal peptide. Some or all of the novel epitopes described here may be useful for monitoring autoimmune human $CD8^+$ T-cells in type 1 diabetic patients and individuals at risk of developing type 1 diabetes. Moreover, our study demonstrates that naturally processed epitopes can be identified with high efficiency, using a combination of predictive algorithms (especially SYFPEITHI), in vitro biochemical assays, and HLA class I–humanized transgenic mice.

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