Major Histocompatibility Complex Class II Molecules Function as a Template for the Processing of a Partially Processed Insulin Peptide into a T-Cell Epitope

Ying Lang, Frederique Forquet, Edwin Speck, Janice Blum, and Terry L. Delovitch

Our understanding of how an autoantigen is processed and presented during the development of a major histocompatibility complex (MHC) class II–dependent and T-cell–mediated autoimmune disease, such as IDDM, is incompletely understood. We have used insulin as a model autoantigen in IDDM to address the question of whether MHC class II molecules play a role in the generation and/or preservation of an autoantigen peptide that stimulates T-cell activation. Analyses of the requirement of I-A^d class II molecules in the processing of the partially processed porcine insulin peptide A1-A14/B1-B16 demonstrate that the binding of this peptide to I-A^d is essential for it to be further processed and tailored into a T-cell epitope. Based on our observations, we propose a two-step model for insulin processing in which insulin is first processed by an enzyme(s) into an intermediate peptide that binds to class II and then class II functions as a template to guide the processing of this partially processed peptide by cathepsin D into a T-cell epitope. Our data further underscore the important realization that MHC class II–directed processing of an autoantigen (e.g., insulin) may regulate *1*) the relative immunodominance of T-cell determinants in an autoantigen, *2*) the self-reactivity to cryptic T-cell epitopes in autoantigens, and *3*) the susceptibility to autoimmune disease. *Diabetes* 45:1711–1719, 1996

From the Autoimmunity/Diabetes Group, The John P. Robarts Research Institute, and the Department of Microbiology and Immunology, University of Western Ontario (Y.L., F.F., E.S., T.L.D.), London, Ontario, Canada; and the Department of Microbiology and Immunology (J.B.), Indiana University, Indianapolis, Indiana.

Address correspondence and reprint requests to Dr. Terry L. Delovitch, Director, Autoimmunity/Diabetes Group, Sheldon H. Weinstein Scientist in Diabetes, The John P. Robarts Research Institute, London, Ontario N6G 2V4, Canada.

Received for publication 21 March 1996 and accepted in revised form 27 June 1996.

APC, antigen-presenting cell; CRPMI, complete RPMI 1640 medium; FCS, fetal calf serum; HEL, hen egg lysozyme; IDE, insulin-degrading enzyme; IL-2, interleukin-2; mAb, monoclonal antibody; MHC, major histocompatibility complex; OVA, ovalbumin; PBS, phosphate-buffered saline; PI, porcine insulin; PMSF, phenylmethylsulfonyl fluoride; TCR, T-cell receptor.

The T-cell receptor (TCR) on CD4$^+$ T-cells generally recognizes antigenic peptides of ~12–19 amino acids in length bound to self class II major histocompatibility complex (MHC) glycoproteins on the surface of an antigen-presenting cell (APC) (1). The formation of such a peptide-MHC complex requires antigen processing and mediates antigen presentation by an APC (2). Exogenous antigens are endocytosed by APCs and are processed by denaturation or proteolysis into peptides in endosomes and lysosomes. Newly synthesized class II molecules travel through the *trans*-Golgi network and reach early endosomes or lysosome-related vesicles before reaching the cell surface. Interaction of a peptide with class II seems to occur in distinct MHC class II–specific intracellular "loading" vesicles, which contain processed peptides and proteases of the endocytic pathway (3). Aspartyl proteases, such as the cathepsins D and E, as well as cysteine proteases, such as cathepsin B, have been implicated in the processing of several antigens (4–6). Current evidence suggests that an endosomal acidic environment facilitates the interaction between a peptide and class II. The binding of a peptide to class II can induce a stable conformation that promotes the transport to and accumulation of these molecules at the cell surface (7,8).

Protein antigens are exposed to endoproteinase and exoproteinase proteolytic activity as they traverse the endocytic pathway. What ensures that some antigenic peptides are rescued from destruction and preserved for recognition by T-cells? Several observations suggest a role for class II molecules in the generation and/or preservation of antigenic peptides. First, APCs of different class II haplotypes can display different T-cell epitopes of the same antigen (9–11), consistent with the notion that polymorphism of MHC molecules may influence antigen presentation. Second, whereas class II molecules bind and present an exogenously added synthetic peptide and its analogs that differ in length and antigenic potency, naturally processed peptides appear to be of rather limited diversity (1,12). An explanation for this limited diversity is that a folded protein antigen may initially be unfolded and/or partially processed to enable a partially processed peptide to interact with a class II molecule. It has been hypothesized that after such an inter-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025

action, further trimming of the partially processed peptide may occur to permit optimum binding to the peptide binding "groove" of class II and the TCR (13). This hypothesis suggests that an MHC class II molecule can influence the processing of a bound peptide.

Indeed, a class II molecule can bind and present a large peptide that extends beyond its peptide binding groove opened on both sides, and the binding of such a peptide to class II protects it from further degradation (14,15). Once a peptide binds to class II, it is very resistant to endoproteases and only slightly resistant to exoproteases. This suggests that a peptide bound to class II can be protected from proteolysis and further trimmed at its NH_2- and/or COOH-terminus to increase its relative affinity for class II and/or the TCR (16). It is interesting to note that MHC class I molecules appear to protect high-affinity peptides generated intracellularly from further processing (17). Based on these types of observations, models have been proposed to explain how class I (18,19) and class II MHC molecules (12) may influence cellular peptide production and maintenance. Essentially, these models allow for either a native antigen to bind to MHC and then be processed into a T-cell epitope or a partially processed peptide to bind to MHC and then be further processed by the same or different enzyme(s).

Further consideration of these models is important for our understanding of how an autoantigen is processed and presented during the development of an MHC class II–dependent and T-cell–mediated autoimmune disease, such as IDDM. A test of these models requires the precise definition of the role of a class II molecule in antigen processing and identification of the enzymes involved in the processing of an autoantigen. Insulin is the only known IDDM autoantigen that is islet β-cell specific, and oral (20,21) or subcutaneous (22) administration of insulin protects from IDDM in the nonobese diabetic mouse model of IDDM. In the present study, we have analyzed the requirement for MHC class II molecules in the processing of porcine insulin (PI). We provide evidence that is consistent with the hypothesis that class II molecules serve as a template to direct the processing of insulin into a T-cell epitope.

RESEARCH DESIGN AND METHODS

Antigens, monoclonal antibodies (mAbs), and reagents. Monocomponent zinc-free crystalline PI was generously provided by Connaught Novo (Toronto, ON). The PI peptide A1-A14/B1-B16 was prepared by 0.25% (wt/wt) chymotrypsin (Worthington) digestion (7 h, 37°C) of PI, as described (23). The ovalbumin (OVA 323-339) peptide was kindly provided by Dr. T. Watts (Department of Immunology, University of Toronto, Toronto, ON). Both the purified I-A^d and I-A^k molecules and the two control peptides 19K MT 1-15 and HIV gp17 65-80, which bind specifically to I-A^d and I-A^k (24), respectively, were kindly provided by Dr. A. Sette (Cytel, San Diego, CA). The murine monoclonal antibodies (mAbs) MK-D6 (IgG2a, κ) specific for I-A^d (25) and 14-4-S (IgG2a, κ) specific for I-$E^{k,d}$ (26) were used in the antigen-processing experiments. Affinity-purified mouse cathepsin D was prepared as described (27). Antipain, E-64, and leupeptin were purchased from Sigma (St. Louis, MO) and dissolved in phosphate-buffered saline (PBS), pH 5.5, immediately before use. Pepstatin (Sigma) was dissolved in DMSO and diluted 30-fold in PBS to avoid cellular toxicity.

Cell lines. The PI/I-A^d specific B8/C3X/B^- (B8) T-cell hybridoma, its B8.P4.10 subclone, and the interleukin-2 (IL-2)-dependent CTL.L T-cell line were used, as reported (23). The OVA 323-339 peptide/I-A^d–specific DO-11.10 T-cell hybridoma (28) was kindly supplied by Dr. T. Watts. The TA3 (expresses surface I-$A^{d/k}$ and I-$E^{d/k}$) murine B-cell hybridoma, M12.4.1 (H-2^d) B-cell lymphoma and M12.C3 MHC class II–negative variant of M12.4.1 cell lines were used, as described (23,29). The MK-D6 and 14-4-4S B-cell hybridomas were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in complete RPMI 1640 medium (CRPMI) supplemented with 5% fetal calf serum (FCS), 2 mmol/l L-glutamine, 25 mmol/l HEPES, pH 7.3, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µmol/l 2-mercaptoethanol. CTL.L cells were maintained with CRPMI plus IL-2 (15 U/ml).

Peptide-MHC class II binding assay. Peptides (10–100 nmol/l) labeled with ^{125}I by the chloramine T method (30) were incubated with affinity-purified MHC class II molecules (2–10 µmol/l) for 48 h at 23°C in PBS containing 1% NP-40, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 200 mmol/l 1,10-phenanthroline (Sigma), pepstatin A (50 µg/ml), 50 mmol/l iodoacetamide, EDTA (3 mg/ml), and NaN_3 (0.1%). Peptide–class II complexes were separated from free peptide by Sephadex G-50 gel filtration during elution in PBS/0.5% NP-40, and the fraction of peptide bound to the I-A^d and I-A^k class II molecules was calculated, as described (16).

Processing of PI and PI peptide A1-A14/B1-B16 by a B-cell APC lysate. TA3 B-cells were lysed by hypo-osmotic shock in PBS containing 0.05 mol/l NaCl, at pH 5.5 or 7.3, and then further homogenized with a tight Dounce homogenizer at 4°C. The lysate (6×10^8 cell equivalents/ml) was spun (8,800*g*, 15 min) to remove nuclei, and membranes in the supernatant were removed by further centrifugation (133,000*g*, 25 min). The resultant TA3 lysate was enriched in cytosolic proteins.

When PI was processed in the presence of fixed TA3 APCs, PI (400 µg/ml in PBS, pH 5.5 or 7.3) was incubated for various times at 37°C in a reaction mix (100-µl vol) containing TA3 lysate (3×10^8 cell equivalents/ml) and fixed TA3 cells (10^6). After washing with CRPMI, pH 7.3, the TA3 cells were co-cultured with B8.P4.10 T-cells in a T-cell activation assay. Alternatively, when PI was processed in the absence of fixed TA3 cells, PI (1 mg/ml) was incubated for 1 h at 37°C with TA3 lysate at pH 5.5. Samples were fractionated by 1 mol/l acetic acid elution from Sephadex G-50, and peptides with a M_r less than or equal to PI were collected, lyophilized, and further resolved by C18 reverse-phase HPLC using an acetonitrile gradient (23). Seven fractions were collected, lyophilized, re-dissolved in CRPMI, and then tested for their capacity to stimulate IL-2 production by PI/I-A^d–specific T-cells in the presence of either fixed or unfixed TA3 APCs during a 48-h T-cell activation assay.

For processing of PI peptide A1-A14/B1-B16 in the presence of fixed APCs, this peptide (10–40 µmol/l) was incubated with fixed APCs (10^6) for 4 h at 37°C in PBS, pH 5.5. After washing with PBS, pH 5.5, the APCs were incubated (100-µl vol) with TA3 lysate (2×10^8 cell equivalents/ml) for various times at 37°C, washed with CRPMI, and co-cultured with B8.P4.10 T-cells in a 48-h T-cell activation assay. In the mAb inhibition assays, fixed APCs (10^6) were preincubated with either the MK-D6 mAb (10 µg/ml) or 14-4-4S ascites (different dilutions) for 90 min. The APCs were then pulsed for 4 h with peptide PI A1-A14/B1-B16 (10 µmol/l), washed (PBS, pH 5.5), incubated with TA3 cell lysate for 16 h at 37°C, further washed, and assayed for T-cell activation. For processing of PI peptide A1-A14/B1-B16 in the absence of fixed APCs, this peptide (40–160 µmol/l) was incubated with TA3 lysate (pH 5.5) for 1–16 h at 37°C and CRPMI was added to stop the reaction. The processed peptide (10–40 µmol/l) was incubated for 4 h with fixed TA3 APCs (10^6) in PBS, pH 7.3, and the washed (CRPMI) APCs were co-cultured with B8.P4.10 cells for 48 h in a T-cell activation assay.

Processing of PI and PI peptide A1-A14/B1-B16 by cathepsin D. Peptide A1-A14/B1-B16 (40 µmol/l) was incubated for 4 h at 37°C with fixed TA3 cells in citrate buffer (20 mmol/l citrate acid, 100 mmol/l KCl, pH 5.5). Cells were washed, resuspended in citrate buffer, and further incubated with 2% (wt/wt) cathepsin D for 30 min at 37°C. In control experiments, the same amount of cathepsin D was reacted for 30 min with pepstatin (0.1 mmol/l), an inhibitor of cathepsin D, before processing of the peptide. The cells were then washed with CRPMI and used in a T-cell activation assay. Alternatively, fixed TA3 cells (10^6) were incubated for 0.5–1.0 h at 37°C with PI (0.9 mg/ml) and 1% (wt/wt) cathepsin D in citrate buffer, washed, and assayed for their ability to stimulate T-cell activation.

APC fixation and T-cell activation. APCs were fixed for 35–40 s with 0.05% glutaraldehyde and checked for their extent of fixation by [4,5-^{3}H]leucine or [^{3}H]TdR (Amersham Canada, Oakville, ON) incorporation (23). B8.P4.10 T-cells (5×10^4/well) were cultured with APCs (2.5×10^4 fixed APCs/well or 5×10^3 unfixed APCs/well) in triplicate 96-wells for 48 h at 37°C in CRPMI. In some experiments, fixed APCs were either preincubated with hen egg lysozyme (HEL, 1 mmol/l), washed, and then reacted with PI peptide A1-A14/B1-B16 or directly pulsed with the latter peptide at the indicated concentrations for 4 h in PBS, pH 5.5. APCs were centrifuged, washed with CRPMI, and incubated with unfixed TA3 cells (at different ratios of fixed:unfixed APCs) and B8 T-cells. The supernatant was also incubated with TA3 APCs and B8 T-cells to monitor the presence of residual PI peptide

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025

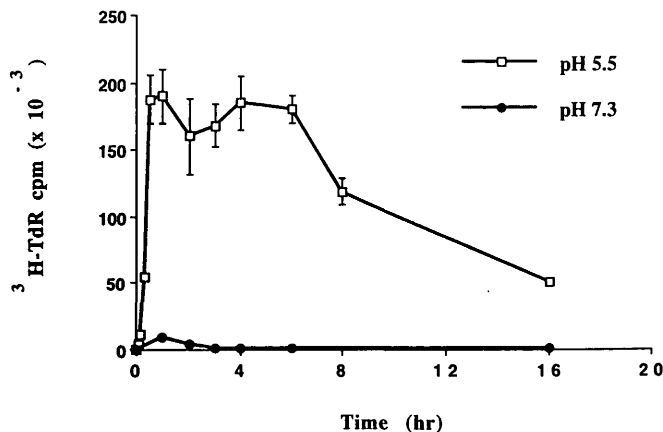

FIG. 1. Insulin is processed into a T-cell epitope at an acidic but not neutral pH in the presence of MHC class II bearing fixed APCs. PI (400 μg/ml) was processed for various times (0–16 h) by a TA3 cell lysate at either pH 5.5 or 7.3 in the presence of fixed TA3 APCs. The APCs were then washed three times, co-cultured with PI/I-A^d–specific B8.P4.10 T-cells, and assayed for IL-2 secretion. Data are the mean [^{3}H]TdR cpm incorporated by triplicate samples ± SD and are representative of three separate experiments.

in the supernatant after incubation with fixed APCs in a 48-h APC assay. Culture supernatants (50 μl) were tested for IL-2 activity on CTL.L cells (10^4) in a 24-h assay, as determined by [^{3}H]TdR incorporation (23). Results are expressed as the mean of triplicates ± SD.

RESULTS

PI is processed into a T-cell epitope at pH 5.5 in the presence of MHC class II–bearing fixed APCs. We previously showed that PI is internalized by pinocytosis and recycled to the surface membrane of a TA3 B-cell APC (31,32). During recycling, PI is enzymatically processed, enabling it to bind to I-A^d class II molecules and to be presented to PI/I-A^d–specific T-cells (23). In the present study, the requirement for class II in antigen processing was analyzed by using fixed TA3 APCs as a source of I-A^d molecules and a TA3 cytosolic lysate as a source of intracellular processing enzymes. Because PI can be proteolytically cleaved at the cell surface and in endosomes of TA3 cells (33), we compared the efficiency of processing of PI at pH 7.3 and at pH 5.5. In the presence of class II I-A^d, PI was rapidly processed at pH 5.5 but not 7.3 into an immunogenic T-cell epitope (Fig. 1); a plateau was reached after 1 h and was maintained for up to 6 h. Thus, PI is processed into a T-cell epitope in the presence of I-A^d–positive APCs at an acidic but not neutral pH, in support of the proposed role of class II molecules and a late endosomal enzyme(s) in antigen processing and presentation (34).

Processing of PI by a TA3 lysate in the absence of MHC class II–bearing fixed APCs does not result in a T-cell epitope. Figure 1 demonstrated that processing of PI by TA3 APC-derived enzymes in the presence of I-A^d–positive fixed TA3 cells resulted in T-cell activation. However, this experiment did not indicate whether PI was first proteolysed by a TA3 enzyme(s) into a T-cell epitope that then bound MHC class II or whether PI was first cleaved into a partially processed peptide that was further processed into a T-cell epitope after binding to class II on fixed TA3 cells. To distinguish between these two possibilities, the capacity of PI processed by TA3 lysate in the absence of fixed TA3 cells to stimulate T-cell activation was tested. Processed PI peptides with a M_r less than or equal to PI were resolved on Sephadex G-50 and further fractionated by reverse-phase HPLC. Of the seven fractions collected (Fig. 2*A*), only fraction 5, which co-elutes with intact PI, elicited T-cell activation when incubated with unfixed TA3 cells (Fig. 2*B*). No T-cell activation was observed after incubation of the peptide fractions with fixed APCs (Fig. 2*B*). These results indicate that *1*) a T-cell epitope of PI capable of being presented by fixed APCs is not produced in the absence of an APC, and *2*) an immunogenic PI peptide is produced only when PI is processed by an APC-derived enzyme(s) in the presence of APCs.

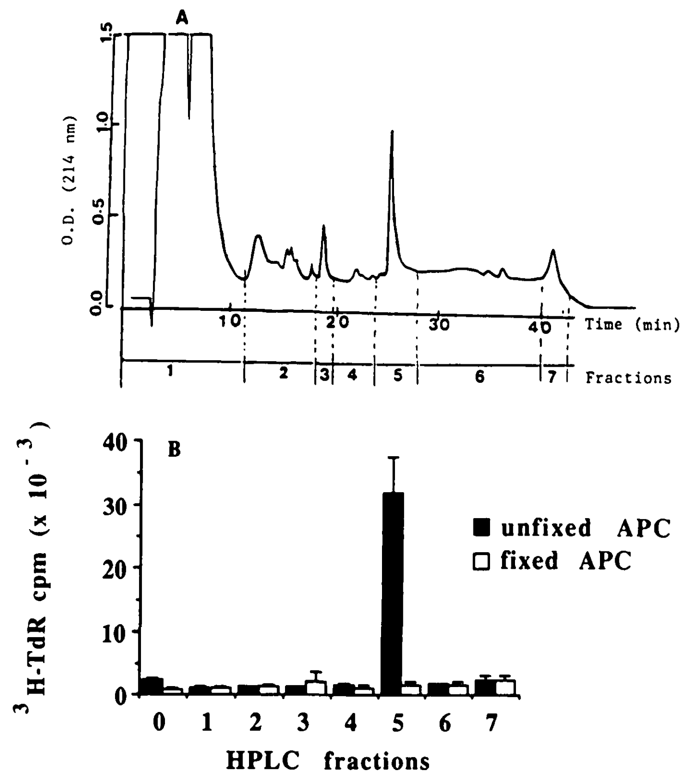

FIG. 2. Processing of PI in the absence of I-A^d–positive cells does not result in T-cell activation. PI (1 mg/ml) was processed for 1 h by a TA3 lysate in the absence of fixed TA3 cells. Peptides with a M_r less than or equal to PI were separated by Sephadex G-50 filtration and further resolved by C18 reverse-phase HPLC. Seven fractions were collected and assayed for their ability to stimulate IL-2 secretion by B8.P4.10 T-cells. *A*: HPLC chromatogram. Retention time (min) and the seven pooled fractions are indicated. *B*: [^{3}H]TdR incorporation by CTL.L cells after the stimulation of B8.P4.10 T-cells in the presence of the seven fractions shown in *A*. Data are the mean [^{3}H]TdR cpm incorporated by triplicate samples ± SD and are representative of three separate experiments.

Processing of PI peptide A1-A14/B1-B16 by a TA3 cell lysate after its incubation with I-A^d–bearing fixed APCs results in a T-cell epitope. Data presented above (Figs. 1 and 2) raise the possibility that a PI T-cell epitope is produced in two sequential steps. First, PI may be cleaved in the absence of class II into a partially processed intermediate peptide. Second, this intermediate peptide may then be further trimmed into a T-cell epitope after binding to class II. Because intact PI does not

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025

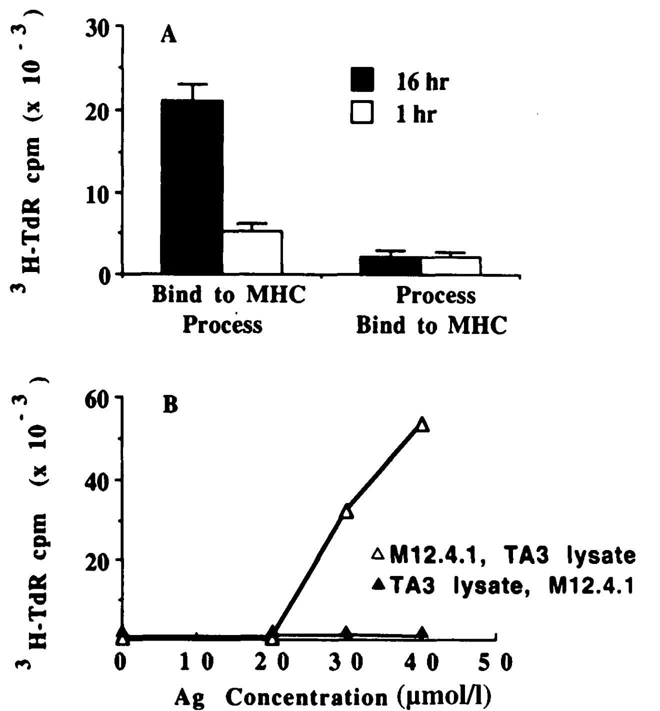

FIG. 3. Binding of PI A1-A14/B1-B16 to I-A^d–positive cells and subsequent processing by a TA3 lysate stimulates T-cell activation. *A*: PI A1-A14/B1-B16 was first incubated with fixed TA3 cells, then processed with a TA3 lysate and assayed for T-cell activation (bind to MHC, process). Alternatively, PI A1-A14/B1-B16 was first processed by a TA3 cell lysate, then incubated with fixed TA3 cells and assayed for T-cell activation (process, bind to MHC). PI A1-A14/B1-B16 was processed for 1 h or 16 h. Background (no peptide) [^{3}H]TdR incorporation was 1,400 cpm. *B*: PI A1-A14/B1-B16 was first incubated with fixed M12.4.1 cells and then processed by a TA3 lysate or was processed by a TA3 lysate and then incubated with M12.4.1 cells. Results are expressed as the mean [^{3}H]TdR cpm incorporated by triplicate samples ± SD and are representative of three experiments.

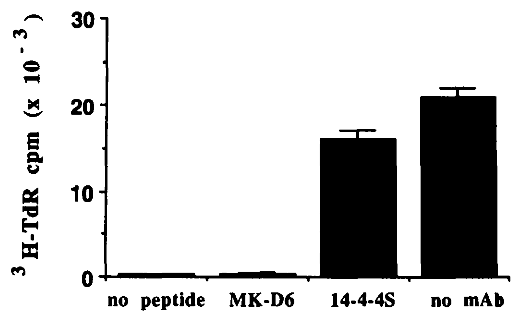

FIG. 4. Presentation of PI A1-A14/B1-B16 is I-A^d dependent. Fixed TA3 cells were preincubated with either the MK-D6 (anti–I-A^d, 10 µg/ml) or control 14-4-4S (anti–I-E$^{k/d}$, used as ascites at a 1:100 dilution) mAb for 90 min and were then exposed to PI A1-A14/B1-B16 and TA3 lysate. Control experiments in which either no PI peptide or no mAb was added to the cultures are also shown. T-cell activation was assayed as in Fig. 3. Results are expressed as the mean [^{3}H]TdR cpm incorporated by triplicate samples ± SD and are representative of three separate experiments.

bind to class II and must be processed to bind class II and be presented by a fixed APC (31), the first step of PI processing presumably is a prerequisite for a PI-derived peptide to bind to class II molecules. To test whether *1*) class II can bind a partially processed PI peptide and direct the further processing of the bound peptide into a T-cell epitope and *2*) the binding of a partially processed PI peptide to class II is essential for its further processing into a T-cell epitope, we analyzed the requirements for the conversion of the partially processed PI peptide A1-A14/B1-B16 into a T-cell epitope. This PI peptide was selected for analysis, since it binds to I-A^d and is presented to PI/I-A^d–specific T-cells by unfixed but not fixed APCs, indicating that it needs further processing to activate T-cells (23,31). A TA3 lysate was used to process this peptide either before or after its incubation with I-A^d–positive APCs, and the ability of the processed peptide to stimulate T-cell activation was then assayed. When PI peptide A1-A14/B1-B16 was processed after prior incubation with I-A^d–positive APCs, a T-cell epitope was generated that activated T-cells (Fig. 3*A*). Significant T-cell activation was detected after 15 min and was enhanced considerably after 16 h of processing. No T-cell activation was observed when the PI peptide was first processed by a TA3 lysate and then added to fixed APCs (Fig. 3*A*). Similar results were obtained using M12.4.1 (surface I-A^d– and I-E^d–positive) B-cells as APCs (Fig. 3*B*). Thus, PI A1-A14/B1-B16 is further processed into an immunogenic peptide only after its incubation with fixed I-A^d–positive APCs.

To identify whether I-A^d or I-E^d molecules mediate the presentation of PI into a T-cell epitope, fixed TA3 APCs were preincubated with either the MK-D6 I-A^d–specific or 14-4-4S I-E^{d,k}–specific mAb for 90 min and then exposed to the PI peptide A1-A14/B1-B16 and TA3 lysate. After 16 h, the APCs were washed and co-cultured with B8.P4.10 T-cells. T-cell activation was extensively inhibited when fixed TA3 APCs were pretreated with the MK-D6 mAb, but was only slightly reduced when these APCs were pretreated with the control 14-4-4S mAb (Fig. 4). The latter result is probably due to nonspecific inhibition, since the 14-4-S mAb was used in ascites form at a dilution of 1:100. These mAb inhibition experiments confirm that a T-cell response to PI peptide A1-A14/B1-B16 is restricted by I-A^d (23,31). The MHC restriction for the presentation of this PI peptide was also determined by performing a binding assay using purified I-A^d molecules. Table 1 shows that the PI peptide A1-A14/B1-B16 binds to I-A^d but not to I-A^k molecules. Two control peptides, 19K MT 1-15 and HIV gp17 65-80, which bind specifically to I-A^d and I-A^k (24), respectively, were used to monitor the specificity of the assay.

PI peptide A1-A14/B1-B16 is processed when bound to I-A^d. Although PI peptide A1-A14/B1-B16 requires the presence of I-A^d–positive cells to be processed, we analyzed whether during processing this peptide is bound to I–A^d and/or other surface molecules on fixed I-A^d–positive APCs. Fixed M12.4.1 or M12.C3 cells were pretreated with HEL (I-E^k–restricted) at pH 5.5 to block nonspecific binding and were further incubated with PI A1-A14/B1-B16. The APC supernatants were then examined for their content of residual stimulatory PI peptide A1-A14/B1-B16 by assaying their capacity to stimulate T-cell activation. Preincubation of the PI peptide with fixed Ia^d-positive M12.4.1 cells, but not the Ia$^-$ variant M12.C3 cells, removed the ability of this peptide to be presented by

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025

TABLE 1
Binding of radiolabeled PI peptide A1-14/B1-16 to I-A^d

Labeled peptide	Percentage of total offered peptide bound to MHC class II	
	A^d	A^k
PI A1-14/B1-16	8.1	0.2
19K MT 1-15	10.5	0.1
HIV gp17 65-80	0.2	14.2

^{125}I labeled peptides (0.2–0.6 µmol/l) were incubated (48 h, 37°C) with affinity purified I-A^d or I-A^k molecules (40 µmol/l) in PBS containing 1% NP-40, 1 mmol/l PMSF, 200 mmol/l 1,10-phenanthroline, 50 µg/ml pepstatin A , 50 mmol/l iodoacetamide, 3 mg/ml EDTA, and 0.1% NaN_3. Peptide-MHC class II complexes were separated from free peptide by elution from Sephadex G-50 in PBS/0.5% NP-40. The fraction of peptide bound to the I-A^d and I-A^k molecules relative to the total amount of offered peptide was calculated.

TA3 cells (Fig. 5*A*). Because these two cell lines apparently differ only in their surface expression of Ia^d molecules (35), these data suggest that PI peptide A1-A14/B1-B16 binds to Ia^d. To confirm this point, fixed Ia^d-positive M12.4.1 cells and the Ia$^-$ variant M12.C3 cells were preincubated with either the MK-D6 anti-I-A^d or 14-4-4S anti-I-E^{d,k} mAb before adding the PI peptide. Presentation of this peptide by TA3 cells was detected after preincubation with the MK-D6 mAb but not 14-4-4S mAb (Fig. 5*B*). In related experiments, co-culture of unfixed TA3 cells and B8 T-cells with fixed M12.4.1 cells that were prepulsed with either a low (40 µmol/l) or high (280 µmol/l) dose of PI A1-A14/B1-B16 did not elicit T-cell activation (Y.L., F.F., E.S., J.B., T.L.D., unpublished observations). These observations clearly show that I-A^d molecules mediate the binding, further processing, and presentation of the PI A1-A14/B1-B16 peptide to T-cells.

Inhibition of PI processing by acid protease but not thiol and serine protease inhibitors. We examined which type of enzyme(s) is involved in the processing of PI for T-cell activation by the use of selected protease inhibitors. TA3 lysate was incubated (30 min, 37°C) with either the thiol and serine protease inhibitors, E-64, leupeptin, and antipain, or an acid protease inhibitor, pepstatin, and then used to process PI for 3–8 h in the presence of fixed TA3 cells. Pretreatment of the TA3 lysate with optimal concentrations (1.5 mmol/l) of the thiol and serine protease inhibitors did not reduce and, in fact, slightly enhanced B8.P4.10 PI-specific T-cell activation (Fig. 6*A*), possibly by preventing epitope destruction. In contrast, treatment of this lysate with pepstatin (0.1 mmol/l) completely inhibited T-cell activation (Fig. 6*B*). At this concentration, pepstatin did not inhibit the APC function of fixed TA3 cells, as evidenced by the ability of these cells to efficiently present the OVA 323-339 peptide (Fig. 6*C*). Similar results were obtained after 18–24 h of processing in the presence of these various inhibitors (data not shown). Thus, an acid protease but not thiol or serine protease appears to be involved in the generation of a PI T-cell epitope.

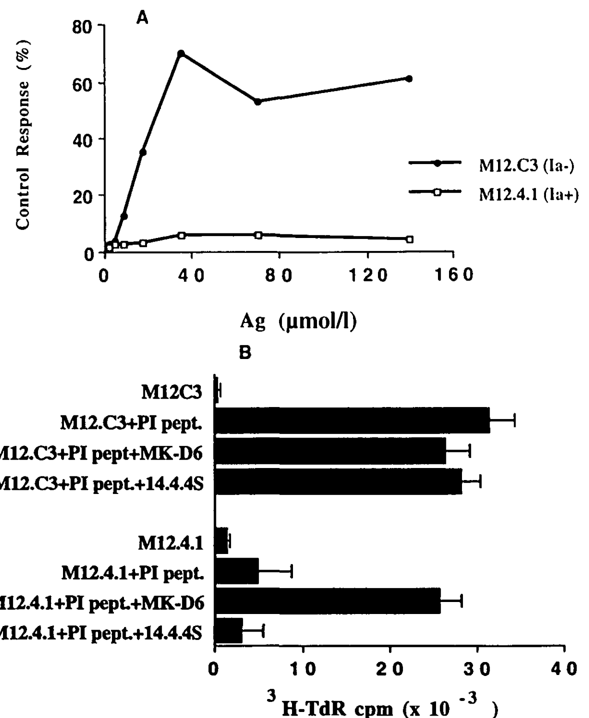

FIG. 5. *A*: binding of PI A1-A14/B1-B16 to surface Ia^d molecules on fixed APCs. Fixed M12.4.1 or M12.C3 cells (5 × 10^6) were incubated for 4 h at 37°C with 1 mmol/l HEL in PBS, pH 5.5, washed three times with the same buffer, and then reacted with the PI A1-A14/B1-B16 peptide (280 µmol/l) in PBS, pH 5.5, in a total volume of 150 µl. After a further 4-h incubation, the cells were pelleted, and the supernatants obtained from M12.4.1 or M12.C3 cells were used at various dilutions in a 48-h T-cell activation assay. *B*: binding of PI A1-A14/B1-B16 to surface I-A^d molecules. The experiment was performed as in *A*, with the exception that the fixed Ia^d-positive M12.4.1 cells and the Ia$^-$ variant M12.C3 cells were preincubated with either the MK-D6 anti-I-A^d (10 µg/ml) or the 14-4-4S anti-I-E^{d,k} mAb (used as ascites at a 1:100 dilution) before adding the PI peptide. Results in *A* are expressed as the percent of control response and are representative of three separate experiments. The maximum control response obtained when unfixed TA3 cells (5 × 10^3) were incubated with B8 T-cells (5 × 10^4) in the presence of the PI peptide A1-A14/B1-B16 (40 µmol/l) was 34,285 cpm in *A* and 31,280 cpm in *B*.

Processing of the A1-A14/B1-B16 PI peptide, but not PI, by cathepsin D results in more efficient T-cell activation. To further investigate the role of an acid protease in PI processing, PI or PI peptide A1-A14/B1-B16 was processed by an excess of purified mouse cathepsin D, an enzyme previously implicated in antigen processing (44–46). Processing of PI for 0.5–1.0 h by cathepsin D in the presence of fixed TA3 cells did not stimulate T-cell activation (Fig. 7*A*). When PI A1-A14/B1-B16 was first bound to fixed TA3 cells during 4 h and then processed for 0.5 h by cathepsin D, more efficient B8.P4.10 T-cell activation was observed than under conditions in which the peptide was not further processed by cathepsin D (Fig. 7*B*). This enhanced T-cell activation was significantly decreased when cathepsin D was treated with 0.1 mmol/l pepstatin for 30 min before incubation with the peptide. These observations suggest that cathepsin D cleaves the PI A1-A14/B1-B16 peptide after the peptide binds to I-A^d, and this additional processing may alter the conformation and/or improve the binding affinity of the resultant PI peptide to I-A^d and/or the TCR.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025

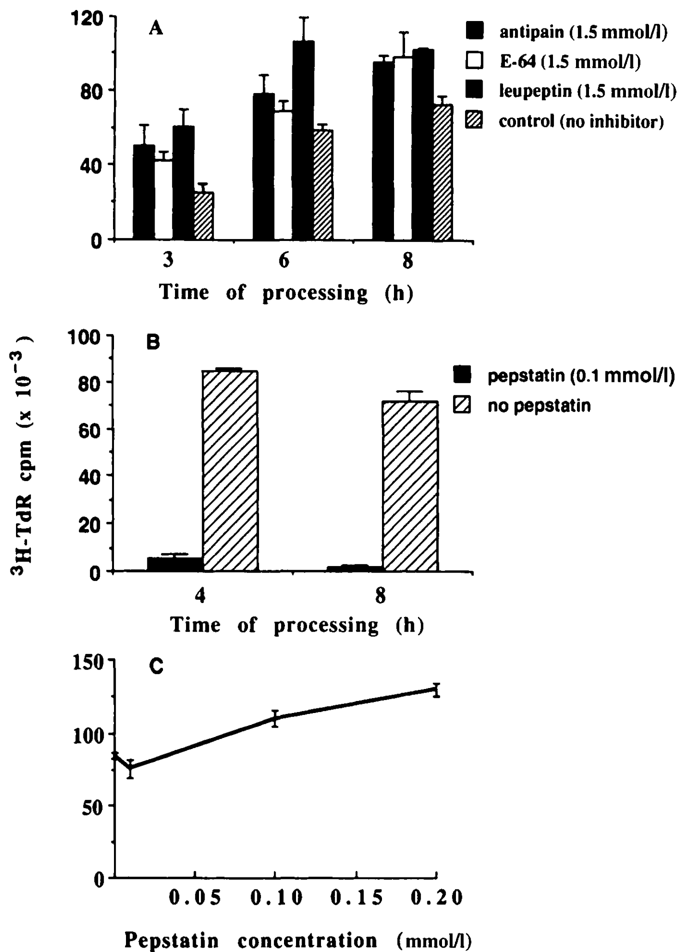

FIG. 6. Influence of protease inhibitors on PI processing and presentation. A TA3 lysate (6 × 10^6 cell equivalents/ml) was treated with either (*A*) the thiol and serine protease inhibitors, antipain, E-64, or leupeptin (each used at 1.5 mmol/l), or (*B*) the acid protease inhibitor, pepstatin (0.1 mmol/l), for 30 min and then incubated with PI (400 µg/ml) in the presence of fixed TA3 cells (10^6) at 37°C. After various times of incubation, the cells were washed three times with CRPMI and co-cultured with B8.P4.10 T-cells. *C*: fixed TA3 cells were incubated with OVA 323-339 (2 µmol/l) for 4 h in the presence of pepstatin (0.01–0.2 mmol/l), washed three times with CRPMI, and then co-cultured with OVA-specific DO-11.10 T-cells in a 48-h T-cell activation assay. Results are expressed as the mean [^{3}H]TdR cpm incorporated by triplicate samples ± SD and are representative of three separate experiments.

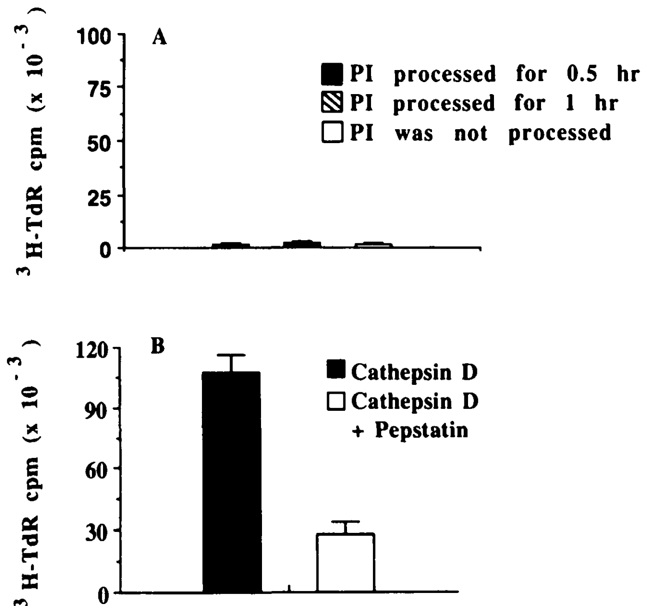

FIG. 7. *A*: processing of PI by cathepsin D. PI (0.9 mg/ml) was or was not (control) processed for 0.5–1.0 h at 37°C with mouse cathepsin D (1%, wt/wt) in the presence of fixed TA3 cells (10^6). The cells were then washed three times with CRPMI and assayed for T-cell activation. In the control experiment, fixed TA3 cells were cultured with B8.P4.10 T-cells in the absence of PI and cathepsin D. *B*: processing of PI A1-A14/B1-B16 by cathepsin D. After a 4-h incubation of fixed TA3 cells (10^6) with the PI peptide A1-A14/B1-B16 (40 µmol/l) in citrate buffer, pH 5.5, the cells were washed in this buffer, incubated for another 0.5 h with 2% cathepsin D, cathepsin D plus 0.1 mmol/l pepstatin, or buffer alone, further washed, and then assayed for T-cell activation. Results are expressed as the mean [^{3}H]TdR cpm incorporated by triplicate samples ± SD and are representative of three experiments. Background cpm (12,250 cpm), observed when the TA3 cells were incubated in the citrate buffer alone, were subtracted from the values shown.

DISCUSSION

This study demonstrates that MHC class II molecules may function as a template in the processing of insulin into an immunogenic peptide for T-cell activation. This conclusion is based on our findings obtained with the partially processed PI A1-A14/B1-B16 peptide, which binds to I-A^d class II molecules but must be further cleaved enzymatically to stimulate T-cell proliferation (23,31). When the PI A1-A14/B1-B16 peptide was first incubated with I-A^d–positive fixed TA3 or M12.4.1 APCs and then treated with a TA3 lysate, used as a source of enzymes, it was processed into an immunogenic T-cell epitope. However, if this peptide was first processed by the TA3 lysate and then bound to class II, no T-cell activation was observed. These data support the hypothesis that the binding of a large intermediate peptide, such as PI A1-A14/B1-B16, to MHC class II is essential for it to be further tailored into a T-cell epitope (13).

Our results suggest that class II molecules serve a dual role in the generation of an immunogenic PI peptide by their ability to sterically control the accessibility of a bound partially processed peptide to an enzyme(s). By analogy to the observation that class I MHC molecules are required to generate peptides of optimal size that bind with higher affinity (17), class II molecules may stabilize the conformation of a T-cell epitope contained within a bound partially processed PI peptide and protect it from proteolytic degradation (14,16). Class II may also guide an enzyme(s) to an exposed region of such an intermediate peptide and enable it to further trim this peptide into a T-cell epitope.

Three possibilities may explain why processing of PI or PI peptide A1-A14/B1-B16 by the TA3 lysate followed by reactivity with fixed APCs did not elicit T-cell activation: the binding of this peptide to class II was competed by a serum protein(s)-derived peptide(s), an inappropriate pH was used for the binding reaction, or the T-cell epitope in this peptide was destroyed. The first two possibilities seem unlikely. T-cell recognition of the OVA 323-339 peptide in association with I-A^d is not inhibited by the processed PI A1-A14/B1-B16 peptide, indicating that there was no detectable A1-A14/B1-B16–derived I-A^d

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025

binding peptides in the digest. An increase in the pH of the binding reaction from 5.5 to 7.3 still resulted in significant T-cell activation. Thus, we favor the third possibility that the T-cell epitope in PI A1-A14/B1-B16 is destroyed during enzymatic processing in the absence of MHC class II–positive APCs. Presumably, in the absence of APCs, PI A1-A14/B1-B16 is not protected from extensive proteolysis and destruction of the T-cell epitope by enzymes present in the lysate. These enzymes may cleave the residues that are essential for MHC class II binding and TCR recognition.

Processing of PI A1-A14/B1-B16 peptide by a TA3 lysate in the presence of fixed M12.4.1 APCs stimulated T-cell activation in an I-A^d–restricted manner. This conclusion was reached based on the ability of the MK-D6 anti–I-A^d mAb but not 14-4-4S anti–I-E^d mAb to inhibit the binding of this PI peptide to I-A^d and thereby enables the M12.4.1 cells to present this peptide to T-cells. These results are consistent with our finding that PI A1-1A14/B1-B16 peptide binds specifically to purified I-A^d molecules (this report) and that I-A^d molecules restrict the presentation of A-chain loop containing PI peptides to T-cells (23). The MK-D6 mAb reacts with an Ab^d-chain hypervariable region 3-related epitope that includes residues 61–71 and 78 (25), which appear to point up from the class II peptide binding groove and would be expected to mediate TCR recognition (15,36). Thus, by binding to I-A^d on M12.4.1 cells, MK-D6 interferes with the interaction between the PI A1-A14/B1-B16 peptide and I-A^d and thereby prevents the consumption of this PI peptide by I-A^d on M12.4.1 cells. These data demonstrate that I-A^d molecules are involved in the binding, further processing, and presentation of the PI A1-A14/B1-B16 peptide.

Peptides bound to MHC class II molecules dissociate from these molecules and, depending on the peptide, either both association and dissociation rates (37) or only the association rate (38,39) is increased at pH 5.5. This raises the possibility that in our experiments, the PI peptide is further processed after it dissociates from class II. We disfavor this possibility based on the following observations. First, a peptide-class II complex is stably expressed for at least 27 h on fixed APCs (40). Second, a peptide bound to class II on fixed APCs cannot be exchanged by a competing peptide, indicating that the bound peptide does not rapidly dissociate from class II on fixed APCs at neutral pH (2). Third, peptide exchange occurs only in metabolically active cells (41). Fourth, T-cell activation did not occur after fixed M12.4.1 APCs were pulsed with the PI peptide, washed, and then incubated with unfixed TA3 APCs and B8 T-cells. We reasoned that if the PI A1-A14/B1-B16 peptide was processed after dissociation from fixed M12.4.1 APCs, then the amount of this peptide subsequently internalized and processed by unfixed TA3 APCs should have been sufficient to activate T-cells. The observation that B8 T-cells were not activated supports the notion that PI A1-A14/B1-B16 is processed while still bound to I-A^d MHC class II molecules.

Cathepsin D, an acid protease found in the same APC endosomal compartments as MHC class II and antigen (3,42), appears to function during antigen processing (4,6,43). Our finding that pepstatin, an aspartyl protease inhibitor, inhibited the ability of a TA3 lysate to process PI and stimulate T-cell activation implicated the involvement of an acid protease in PI processing. This was shown directly by the ability of purified mouse cathepsin D to process the PI A1-A14/B1-B16 peptide, but not intact PI, into a T-cell epitope. These data are consistent with a role for cathepsin D in the cleavage of PI into a T-cell epitope. Cathepsin D hydrolyzes peptide bonds formed between two hydrophobic amino acid residues (44) and would be expected to cleave PI A1-A14/B1-B16 between residues A13-A14, B1-B2, B11-B12, and B15-B16. In this regard, it is interesting to note that PI A1-A14/B7-B15 is presented to PI-specific T-cells more efficiently than PI A1-A14/B1-B16 (23).

The results presented here suggest a model (Fig. 8) in which an enzyme(s) may digest PI into an intermediate peptide that binds to I-A^d, and this intermediate, while bound to I-A^d, is subsequently cleaved by cathepsin D into a T-cell epitope. Insulin-degrading enzyme (IDE), a neutral metalloendoproteinase, is such a candidate enzyme, since it mediates the processing and presentation of PI and generates an A-chain loop–containing intermediate peptide (e.g., A1-A14/B1-B9) that requires additional processing to render it immunogenic (45,46). However, processing of PI in the presence of IDE, cathepsin D, and MHC class II does not yield an immunogenic PI T-cell epitope (Y.L., F.F., E.S., J.B., T.L.D., unpublished observations). It is possible that the combination of IDE and cathepsin D does not produce a T-cell epitope.

Another enzyme, in addition to IDE, may be required during the initial processing of PI into an intermediate peptide that binds to I-A^d. For example, because the reduction of disulfide bonds is required for insulin processing (47,48), a disulfide bond–reducing enzyme may be involved. Glutathione insulin dehydrogenase is such an enzyme that reduces insulin A- and B-chains and renders them susceptible to further degradation by nonspecific hydrolases (31). If disulfide reduction is an essential mechanism of cellular insulin processing, our demonstration that a disulfide-linked PI peptide is able to bind to class II suggests that this reduction may occur after the formation of peptide-class II complexes on the surface of an APC. Heterodimeric disulfide-linked peptides may protect the A-chain free thiol groups from oxidation until interaction with T-cells. It is interesting to note that a reducing environment was recently demonstrated in early endocytic compartments of TA3 B-cells and shown to facilitate the presentation of antigens delivered into this organelle (49). Reduction of the disulfide-linked insulin peptide bound to class II antigens might occur as the complex recycles through such an early compartment. Class II complexes have been shown to recycle in murine and human B-cells (50,51). Thus, the production of a single dominant insulin T-cell determinant may require the combined action of several proteases.

Our results and model of class II–dependent processing *1*) extend and support the hypothesis of "MHC-guided processing" (13), *2*) emphasize the notion that whole denatured proteins and partially processed peptides bind to class II (52–54), and *3*) favor the idea of "determinant capture" in which the binding of an immunodominant

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025

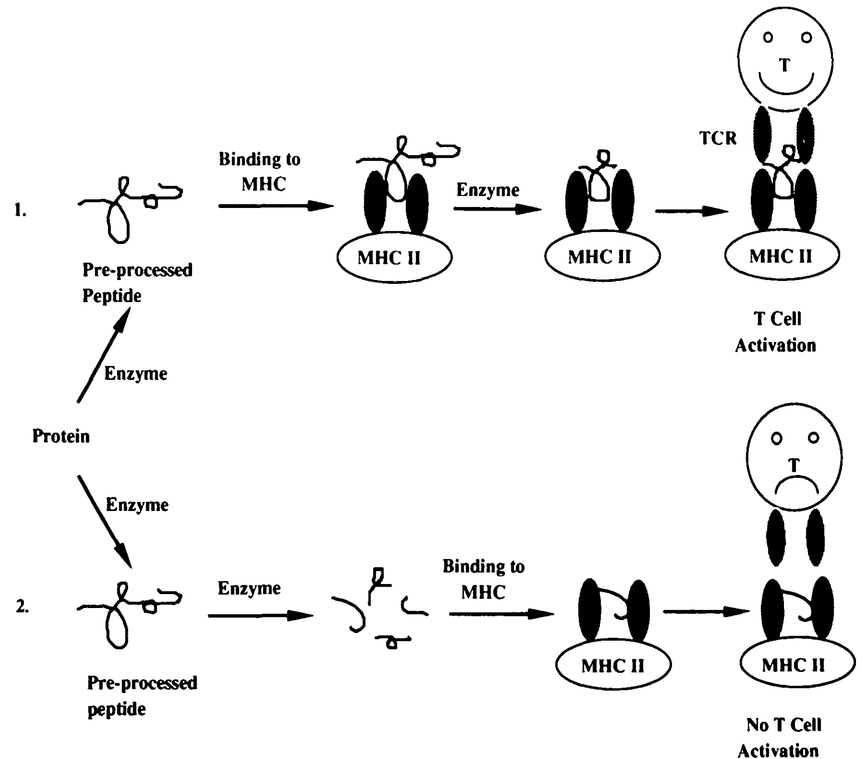

FIG. 8. Model for MHC class II–dependent antigen processing. MHC class II molecules may serve a dual role in the generation of an immunogenic peptide by their ability to sterically control the accessibility of a bound partially processed peptide to an enzyme(s), as indicated in pathway 1. A partially processed peptide binds to a class II molecule with the T-cell epitope in the peptide binding groove and the flanking sequences extending beyond the groove. This binding protects the T-cell epitope from further proteolysis and allows the flanking sequences to be further trimmed. A TCR would be expected to recognize the further processed peptide complexed with a class II molecule. If the same partially processed peptide is further processed in the absence of a class II molecule, as indicated in pathway 2, the T-cell epitope will be destroyed and the resulting peptides may not bind to class II. Alternatively, one of these peptides may bind to class II, but the complexes formed may not be recognized by a TCR.

determinant on an antigenic peptide to one class II molecule captures a neighboring flanking determinant(s) that would otherwise bind to a second class II molecule (13). Thus, MHC class II–directed processing of an autoantigen may significantly influence the relative immunodominance of a set of T-cell determinants in this protein and thereby regulate self-reactivity to cryptic T-cell epitopes in autoantigens and susceptibility to autoimmune disease (13). Evidence for the appearance of immunodominant and cryptic T-cell epitopes of glutamic acid decarboxylase, another IDDM autoantigen, and their role in the regulation of peripheral tolerance and susceptibility to IDDM was recently described (55,56). In the case of insulin, it has been shown that insulin B-chain or various insulin B-chain peptides are recognized by T-cell clones and that treatment of NOD mice with B-chain or these B-chain peptides induces regulatory $CD4^+$ T-cells that mediate protection from IDDM (20–22). Thus, I-A^{g7}–mediated MHC class II–guided processing of insulin into a B-chain T-cell epitope may influence susceptibility to IDDM in NOD mice.

ACKNOWLEDGMENTS

This work was supported by grant MT 5729 from the Medical Research Council of Canada to T.L.D. and grants DK49841 and AI333418 from the National Institutes of Health to J.B. F.F. was the recipient of a postdoctoral fellowship from the Juvenile Diabetes Foundation International.

We thank all of the members of our lab as well as Drs. John Semple, Tania Watts, and Michelle Letarte for their helpful advice and criticism throughout the course of this work; Drs. Tania Watts and Alessandro Sette for generously providing peptides and purified MHC class II molecules; and Drs. Laurie Glimcher, Ethan Shevach, and Michel Pierres for kindly supplying cell lines.

REFERENCES

1. Engelhard VH: Structure of peptides associated with class I and class II MHC molecules. *Annu Rev Immunol* 12:181–207, 1994
2. Cresswell P: Assembly, transport, and function of MHC class II molecules. *Annu Rev Immunol* 12:259–293, 1994
3. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ: Segregation of class II molecules from MHC class II molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349:669–676, 1991
4. Takahashi H, Cease KB, Berzofsky JA: Identification of proteases that process distinct epitopes on the same protein. *J Immunol* 142:2221–2229, 1989
5. Van Noort JM, Boon J, Van der Drift ACM, Wagenaar JPA, Boots AMH, Boog CJP: Antigen processing by endosomal proteases determines which sites of sperm-whale myoglobin are eventually recognized by T-cells. *Eur J Immunol* 21:1989–1996, 1991
6. Bennett K, Levine T, Ellis JS, Peansky R, Samloff MI, Kay J, Chain BM: Antigen processing for presentation by class II major histocompatibility complex requires cleavage by cathepsin E. *Eur J Immunol* 22:1519–1524, 1992
7. Sadegh-Nasseri S, Germain RN: A role for peptide in determining MHC class II structure. *Nature* 353:167–170, 1991
8. Germain RN, Hendrix LR: MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* 353:134–139, 1991
9. Roy S, Scherer T, Briner TJ, Smith AJ, Gefter ML: Murine MHC polymorphism and T-cell specificities. *Science* 244:572–575, 1989
10. Gammon G, Shastri N, Cogswell J, Wilbur S, Sadegh-Nasseri S, Krzych U, Miller A, Sercarz E: The choice of epitopes utilized on a protein antigen depends on multiple factors distant from as well as at the determinant site. *Immunol Rev* 98:53–73, 1987
11. Berzofsky JA, Brett SJ, Streicher HZ, Takahashi H: Antigen processing for presentation to T lymphocytes: function, mechanism, and implications for the T-cell repertoire. *Immunol Rev* 106:5–31, 1988
12. Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA: Sequence analysis of peptides bound to MHC class II molecules. *Nature* 353:622–627, 1991
13. Sercarz EE, Lehmann PV, Ametani A, Benichou G, Miller A, Moudgil K: Dominance and crypticity of T-cell antigenic determinants. *Annu Rev Immunol* 11:729–766, 1993
14. Donermeyer DL, Allen PM: Binding to Ia protects an immunogenic peptide from proteolytic degradation. *J Immunol* 142:1063–1068, 1989
15. Madden DR: The three-dimensional structure of peptide-MHC complexes. *Annu Rev Immunol* 13:587–622, 1995

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/358508/45-12-1711.pdf by guest on 12 May 2025

16. Mouritsen S, Meldal M, Werdelin O, Hansen AS, Buus S: MHC molecules protect T-cell epitopes against proteolytic destruction. *J Immunol* 49:1987–1993, 1992
17. Ojcius DM, Langlade-Demoyen P, Gachelin G, Kourilsky P: Role for MHC class I molecules in selecting and protecting high affinity peptides in the presence of proteases. *J Immunol* 152:2798–2810, 1994
18. Falk K, Rammensee HG: Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature* 348:248–251, 1990
19. Del Val M, Schlicht HJ, Ruppert T, Reddehase MJ, Koszinowski UH: Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. *Cell* 66:1145–1153, 1991
20. Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL: Suppression of diabetes in NOD by oral administration of porcine insulin. *Proc Natl Acad Sci USA* 88:10252–10256, 1991
21. Bergerot I, Fabien N, Maguer V, Thivolet C: Oral administration of human insulin to NOD mice generates CD4$^+$ T cells that suppress adoptive transfer of diabetes. *J Autoimmun* 7:655–663, 1994
22. Muir A, Peck A, Clare-Salzer M, Song YH, Corenelius J, Luchetta R, Krischer J, Maclaren N: Insulin immunization of NOD mice induces a protective insulitis characterized by diminished intraislet interferon-gamma transcription. *J Clin Invest* 95:628–634, 1995
23. Naquet P, Ellis J, Singh B, Hodges RS, Delovitch TL: Processing and presentation of insulin. I. Analysis of immunogenic peptides and processing requirements for insulin A loop-specific T-cells. *J Immunol* 139:3955–3963, 1987
24. Hunt DF, Michel H, Dickinson TA, Shabanowitz J, Cox Al, Sakaguchi K, Appella E, Grey HM, Sette A: Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. *Science* 256:1817–1820, 1992
25. Kappler JW, Skidmore B, White J, Marrack P: Antigen-inducible, H-2 restricted, interleukin-2 producing T-cell hybridomas: lack of independent antigen and H-2 recognition. *J Exp Med* 153:1198–1214, 1981
26. Ozato K, Mayer N, Sachs DH: Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J Immunol* 124:533–540, 1980
27. Blum JS, Diaz R, Diment S, Fiani M, Mayorga L, Rodman JS, Stahl PD: Proteolytic processing in endosomal vesicles. *Cold Spring Harbor Symp Quant Biol* 54:287–292, 1989
28. Shimonkevitz R, Kappler J, Marrack P, Grey H: Antigen recognition by H-2 restricted T-cells. I. Cell-free antigen processing. *J Exp Med* 158:303–316, 1983
29. Glimcher LH, Schroer JA, Chan C, Shevach EH: Fine specificity of cloned insulin-specific T-cell hybridomas: evidence supporting a role for tertiary conformation. *J Immunol* 131:2868–2874, 1983
30. Hunter W, Greenwood F: Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* 194:495–498, 1962
31. Delovitch TL, Semple JW, Bernard NF, Ellis J, Champagne P, Phillips ML: Pathways of processing of insulin by antigen-presenting cells. *Immunol Rev* 106:195–222, 1988
32. Lin J, Berzofsky JA, Delovitch TL: Ultrastructural study of internalization and recycling of antigen by antigen presenting cells. *J Mol Cell Immunol* 3:321–345, 1988
33. Semple JW, Ellis J, Delovitch TL: Processing and presentation of insulin. II. Evidence for intracellular, plasma membrane-associated and extracellular degradation of human insulin by antigen-presenting B-cells. *J Immunol* 142:4184–4193, 1989
34. Jensen PE: Enhanced binding of peptide antigen to purified class II major histocompatibility proteins at acidic pH. *J Exp Med* 174:1111–1120, 1991
35. Glimcher LH, McKean DJ, Choi E, Seidman JG: Complex regulation of class II gene expression: analysis with class II mutant cell lines. *J Immunol* 135:3542–3550, 1985
36. Braunstein NS, Germain NR, Loney K, Berkowitz N: Structurally interdependent and independent regions of allelic polymorphism in class II MHC molecules. *J Immunol* 145:1635–1645, 1990
37. Tampe R, McConnell HM: Kinetics of antigenic peptide binding to the class II major histocompatibility molecule I-A^d. *Proc Natl Acad Sci USA* 88:4661–4665, 1991
38. Jensen PE: Long-lived complexes between peptide and class II major histocompatibility complex are formed at low pH with no requirement for pH neutralization. *J Exp Med* 176:793–798, 1992
39. Lee JM, Watts TH: On the dissociation and reassociation of MHC class II-foreign peptide complexes: evidence that brief transit through an acidic compartment is not sufficient for binding site regeneration. *J Immunol* 144:1829–1834, 1990
40. Harding CV, Roof RW, Unanue ER: Turnover of Ia-peptide complex is facilitated in viable antigen-presenting cells: biosynthetic turnover of Ia vs peptide exchange. *Proc Natl Acad Sci USA* 86:4230–4234, 1989
41. Adorini L, Appella E, Doria G, Cardineaux F, Nagy Z: Competition for antigen presentation in living cells involves exchange of peptides bound by class II MHC molecules. *Nature* 342:800–803, 1989
42. Harding CV, Collins DS, Slot JV, Geuze HL, Unanue ER: Liposome-encapsulated antigens are processed in lysosomes, recycled, and presented to T-cells. *Cell* 64:393–401, 1991
43. Diment S: Different roles for thiol and aspartyl proteases in antigen presentation of ovalbumin. *J Immunol* 145:417–422, 1990
44. van Noort JM, van der Drift ACM: The selectivity of cathepsin D suggests an involvement of the enzyme in the generation of T-cell epitopes. *J Biol Chem* 264:14159–14164, 1989
45. Delovitch TL, Lazarus AH, Phillips ML, Semple JW: Antigen binding and processing by B-cell antigen-presenting cells: influence of T- and B-cell activation. *Cold Spring Harbor Symp Quant Biol* 54:333–343, 1989
46. Semple JW, Lang Y, Speck E, Delovitch TL: Processing and presentation of insulin. III. Insulin degrading enzyme: a neutral metallooendoproteinase that is non-homologous to classical endoproteinases mediates the processing of insulin epitopes for T helper cells. *Int Immunol* 4:1161–1167, 1992
47. Jensen PE: Reduction of disulfide bonds during antigen processing: evidence from a thiol-dependent insulin determinant. *J Exp Med* 1174:1121–1130, 1991
48. Hampl J, Gradehandt G, Kalbacher H, Rude E: In vitro processing of insulin for recognition by murine T-cells results in the generation of A chains with free CysSH. *J Immunol* 148:2664–2671, 1992
49. Gainey D, Short S, McCoy KL: Intracellular location of cysteine transport activity correlates with productive processing of antigen disulfide. *J Cell Physiol* 168:248–254, 1996
50. Harding C, Roof RW, Unanue ER: Turnover of Ia-peptide complexes is facilitated in viable antigen presenting cells: biosynthetic vs peptide exchange. *Proc Natl Acad Sci USA* 86:4230–4234, 1989
51. Reid PR, Watts C: Cycling of MHC class II glycoproteins through a primaquine-sensitive intracellular compartment. *Nature* 346:655–657, 1990
52. Streicher HZ, Berkhower IJ, Busch M, Gurd FRN, Berzofsky JA: Antigen conformation determines processing requirements for T-cell activation. *Proc Natl Acad Sci USA* 81:6831–6835, 1984
53. Lee P, Matsueda GR, Allen PM: T-cell recognition of fibrinogen: a determinant on the Aα chain does not require processing. *J Immunol* 140:1063–1068, 1988
54. Sette A, Adorini L, Colon SM, Buus S, Grey HM: Capacity of intact proteins to bind to MHC molecules. *J Immunol* 143:1265–1267, 1989
55. Kaufman DL, Clare-Salzer M, Tian J, Fforsthuber T, Ting GSP, Robinson P, Atkinson MA, Sercarz EE, Tobin AJ, Lehmann PV: Spontaneous loss of T cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 366:69–72, 1993
56. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO: Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature* 366:72–75, 1993

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/45/12/1711/338508/45-12-1711.pdf by guest on 12 May 2025