

pH-dependent Peptide Binding Properties of the Type I Diabetes-associated I-A^{g7} Molecule: Rapid Release of CLIP at an Endosomal pH

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Summary

MHC class II molecules and invariant chain assemble at a neutral pH in the endoplasmic reticulum and are transported to a low pH compartment where the invariant chain is trimmed to the class II-associated invariant chain peptide (CLIP). For many major histocompatibility complex class II molecules, DM is required for rapid removal of CLIP, which allows binding of antigenic peptides. Since I-A^{g7} confers susceptibility to type I diabetes in NOD mice, the biochemical requirements for peptide loading were examined using soluble I-A^{g7} expressed in insect cells. I-A^{g7} formed long-lived complexes with naturally processed peptides from transferrin and albumin, whereas several peptides that represent T cell epitopes of islet autoantigens were poor binders. I-A^{g7}-peptide complexes were not sodium dodecyl sulfate (SDS) resistant, indicating that SDS sensitivity may be an intrinsic property of I-A^{g7}. Complexes of I-A^{g7} and CLIP formed at a neutral pH, but rapidly dissociated at pH 5. This rapid dissociation was due to a poor fit of M98 of CLIP in the P9 pocket of I-A^{g7}, since substitution of M98 by a negatively charged residue greatly enhanced the stability of the complex. These biochemical properties of I-A^{g7} result in the rapid generation of empty molecules at an endosomal pH and have a global effect on peptide binding by I-A^{g7}.

Key words: major histocompatibility complex • autoimmunity • antigen presentation • I-A^{g7} • type I diabetes

Genome-wide analyses in families with type I diabetes and in nonobese diabetic (NOD)¹ mice demonstrated that multiple genes contribute to susceptibility and that the MHC is an important susceptibility locus (1–5). In humans, susceptibility is associated with the MHC class II region, in particular with the DR4/DQ8 and DR3/DQ2 haplotypes. The risk for the development of type I diabetes is particularly high in subjects who carry both the DR4/DQ8 and DR3/DQ2 haplotype or who are homozygous for either haplotype (1, 6). It is generally thought that the DQ molecules (DQ8 and DQ2) are involved in the disease process, but particular DR4 alleles also contribute to susceptibility (for review see reference 7). Both DQ8 and DQ2 are characterized by the absence of aspartic acid at β 57 (alanine in

both alleles). The DR2/DQ6 haplotype (DQB1*0602) confers dominant protection from the disease.

Two features characterize the MHC class II region of NOD mice. NOD mice express only I-A^{g7} but not I-E molecules, due to a deletion in the E α promoter. This lack of I-E expression is important since transgenic expression of I-E or other I-A molecules protects from spontaneous disease (5, 8–11). The I-A molecule of NOD mice, I-A^{g7}, has the same α chain as I-A^d but a unique β chain sequence. The most striking characteristic of the I-A^{g7} β chain is the presence of histidine and serine at β 56 and β 57, instead of proline and aspartic acid as in other murine I-A alleles (12). Crystallographic studies of human and murine MHC class II molecules demonstrated that β 57 is located in the P9 pocket of the peptide binding site. In most MHC class II molecules, β 57 forms a salt bridge with α 76, which is missing in I-A^{g7} (13–16).

It has recently been proposed that I-A^{g7} molecules are poor peptide binders. This hypothesis is based on the observation that only a small fraction of immunoprecipitated I-A^{g7} molecules are SDS resistant. Also, binding experiments

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¹Abbreviations used in this paper: CLIP, class II-associated invariant chain peptide; HSV, Herpes simplex virus; MBP, myelin basic protein; NOD, nonobese diabetic.

with affinity-purified, detergent-solubilized I-A^{g7} molecules demonstrated little or no binding of peptides in that study (17). To examine the biochemical properties of I-A^{g7} in the absence of detergent, we expressed I-A^{g7} as a soluble protein in *Drosophila* Schneider cells. I-A^{g7} formed long-lived complexes with naturally processed peptides from transferrin and serum albumin, while several peptides that represent T cell epitopes of islet antigens were poor binders. I-A^{g7} formed a complex with the class II-associated invariant chain peptide (CLIP) at a neutral pH, but these complexes rapidly dissociated at pH 5. These biochemical properties of I-A^{g7} result in the rapid generation of empty molecules at the pH of the peptide loading compartment and have a global effect on peptide binding by this MHC class II molecule.

Materials and Methods

Synthetic Peptides. Peptides were synthesized by Quality Controlled Biochemicals, Inc. and subjected to quality control by reverse-phase HPLC and mass spectrometry.

cDNA Constructs. cDNA constructs were made by reverse transcriptase PCR using RNA from NOD spleens. Fos and Jun dimerization domains were attached to the 3' end of the I-A^{g7} α and β extracellular domains, respectively, through a seven-amino acid linker [Val-Asp-(Gly)₅] that contained a Sall restriction site. The cDNAs encoding the signal peptides and extracellular domains of I-A^{g7} α and β chains were amplified first and cloned separately into the EcoRI/Sall site of the pRmHa-3 vector under the control of the inducible metallothionein promoter (18). The Fos and Jun segments were excised with Sall from similar constructs made for HLA-DR2 (19) and cloned into the Sall site of these vectors (I-A α -Fos, I-A β -Jun). The orientation of the Fos and Jun segments and the sequence of the constructs were confirmed by dideoxy-sequencing. The following oligonucleotides were used for the amplification of I-A α and I-A β segments. Forward primer for I-A α , 5' AAA AAA gAA TTC ATg CCg TgC AgC AgA gCT CTg 3'; reverse primer for I-A α , 5' AAA AAA gTC gAC TTC TgT CAg CTC TgA CAT gg 3'; forward primer for I-A β , 5' AAA AAA gAA TTC ATg gCT CTg CAg ATC CCC AgC 3'; reverse primer for I-A β , 5' AAA AAA gTC gAC CTT gCT CCg ggC AgA CTC ggA 3'. The EcoRI and Sall sites in the forward and reverse primers, respectively, are underlined.

Transfection of *Drosophila* Schneider Cells. *Drosophila melanogaster* S2 cells (4×10^6) were transfected with 20 μ g of each plasmid, 0.5 μ g of hygromycin selection vector pUC-hygMT, and 20 μ l of liposomes (Invitrogen) in 1 ml medium without FCS for 4 h. After transfection, cells were grown in Schneider medium (Sigma Chemical Co.) supplemented with 10% of FCS. Selection was performed by addition of hygromycin to a final concentration of 0.1 mg/ml. Approximately 2 wk after transfection, cells were cloned by limiting dilution in 96-well plates. Clones were expanded and tested for secretion of I-A^{g7} by Western blot analysis after induction of expression with copper sulfate (1 mM). The clone with the highest expression level was scaled up in roller bottles using Schneider medium supplemented with 5% FCS and 0.1 mg/ml of hygromycin. The supernatant was harvested 4 d after induction of protein expression with copper sulfate at 1 mM (Sigma Chemical Co.).

Purification of I-A^{g7}. Supernatants were concentrated by ultrafiltration using a YM30 spiral membrane cartridge (Amicon).

The I-A-specific 10-2.16 mAb (20; hybridoma from the American Type Culture Collection) was coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Biotech). The concentrated supernatant was passed through a Sepharose 4B pre-column and the 10-2.16 column at a flow rate of 9 ml/h. The column was then washed with 100 mM phosphate buffer, pH 8.0, and the protein was eluted with 2 bed volumes of 50 mM glycine, pH 11.5. Fractions were immediately neutralized by addition of 2 M Tris, pH 8.8, and dialyzed against PBS. Protein concentration was determined with the Coomassie Plus protein assay (Pierce).

Western Blot Analysis and HPLC Gel Filtration. Antisera specific for the Fos and Jun dimerization domains were generated by immunizing Lewis rats with synthetic peptides (provided by Dr. P. Kim, Whitehead Institute, Cambridge, MA). Fos peptide sequence, Ac-CGGTDTLQAETDQLEDEKYALQTEIANLLKE-KEKL-CONH₂; Jun peptide sequence, Ac-CGGIARLEEKVK-TLKAQNYELASTANMLREQVAQL-CONH₂.

For Western blot analysis, proteins were separated under reducing conditions by SDS-PAGE (12%) and transferred to a polyvinylidene difluoride membrane (Millipore Corp.). Membranes were blocked overnight in 3% non-fat dry milk in TBST-buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween 20). Membranes were probed with Fos antiserum (1:1,000) and/or Jun antiserum (1:500). Bound Fos and Jun antibodies were detected by incubating the membranes with horseradish peroxidase-conjugated anti-rat IgG (1:3,000). The blots were developed by ECL (Amersham).

HPLC gel filtration analysis of I-A^{g7} was performed using a Bio-Gel SEC 30-XL column (300×7.8 mm; Bio-Rad) with phosphate buffered saline, pH 7.8, at a flow rate of 0.8 ml/min. The following proteins were used as molecular mass standards: thyroglobulin (669 kD), aldolase (158 kD), BSA (67 kD), ovalbumin (43 kD), and chymotrypsinogen A (25 kD).

Peptide Binding Assays. Purified I-A^{g7} (0.6 μ g) was incubated with biotinylated test peptide at 37°C for 18 h in a final volume of 50 μ l. Buffers were either 20 mM sodium acetate/100 mM NaCl, pH 5.0, or PBS, pH 7.4, both containing 1 mM PMSF and 1 mM EDTA. In parallel, a 96-well plate (Fluoronunc; Nunc) was coated overnight at 4°C with 50 μ l of 10 μ g/ml of the I-A-specific antibody 10-2.16 in sodium bicarbonate buffer, pH 9.6. Nonspecific binding sites were blocked with 10% FCS in PBS/0.05% Tween 20 (PBS-Tween) for 2 h at room temperature. Wells were washed three times with PBS-Tween and I-A^{g7}-peptide samples were added to the wells. After a 1-h incubation, wells were washed four times with PBS-Tween and europium-labeled streptavidin (Wallac Oy, Finland) was added (1:2,000 in PBS/0.5% BSA, 100 μ l/well) for detection of I-A^{g7}-bound biotinylated peptide. After a 1-h incubation at room temperature, wells were washed six times with PBS-Tween and Delfia enhancement solution (Wallac Oy) was added. Fluorescence was quantitated after 30 min in a fluorescence plate reader (Delfia 1234; Wallac Oy).

For competition assays, 0.6 μ g of soluble I-A^{g7} was incubated with biotinylated transferrin peptide (1 μ M) at pH 5.0 in the presence of nonbiotinylated competitor peptide (8 nM–25 μ M). I-A^{g7}-bound biotinylated transferrin peptide was quantitated as described above.

Peptide Dissociation Experiments. For CLIP and CLIP 98D dissociation assays, soluble I-A^{g7} was loaded with biotinylated CLIP or CLIP 98D (1 μ M) at 37°C for 18 h at pH 7.4. An equal volume of 20 mM sodium acetate, pH 4.5, or PBS, pH 7.4, was added in the presence of nonlabeled mouse serum albumin pep-

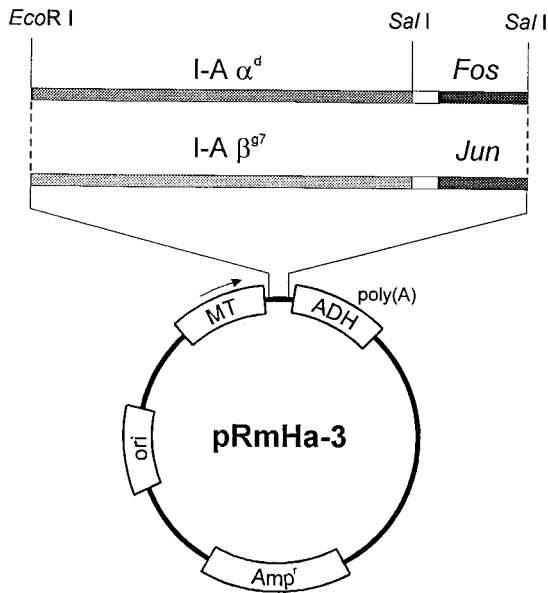


Figure 1. cDNA constructs for the expression of soluble I-A^{g7}. The signal peptides and extracellular domains of I-A^{g7} α and β chains were fused in frame with the leucine zipper dimerization domains of the transcription factors Fos and Jun. A short flexible linker [Val-Asp-(Gly)₅] was placed between the 3' end of the I-A α/β extracellular domains and the Fos/Jun leucine zippers. The I-Aα-Fos and I-Aβ-Jun constructs were separately cloned into the pRmHa-3 vector under the control of the inducible metallothionein promoter. These constructs were transfected into *Drosophila* Schneider cells.

tide (100 μM). The final pH was either 5.0 or 7.4. At different time points (5–120 min), the reaction was stopped by freezing the samples at –20°C in the presence of BSA (200 μM). For quantification of bound biotinylated peptide, samples were mixed with an equal volume of 40 μM Tris, pH 7.4, and captured with 10-2.16 antibody-coated wells at 4°C for 1 h. All wells were developed by incubation for 1 h at 4°C with streptavidin-europium (dilution of 1:2,000). Fluorescence was quantitated as described above.

For other peptide dissociation assays, soluble I-A^{g7} was incubated with biotinylated peptide (1 μM) at 37°C for 18 h at pH 5.0 in a final volume of 50 μl. Then the pH of the samples was either maintained at pH 5.0 or adjusted to pH 7.4 (by addition of Tris, pH 8.8). Nonlabeled mouse serum albumin peptide (100 μM) was added to prevent rebinding of biotinylated peptides. At different

time points (5 min to 72 h), reactions were stopped by freezing the samples at –20°C in the presence of BSA (200 μM). Bound biotinylated peptides were then quantitated as described above.

Herpes Simplex Virus 2 VP16-specific T Cell Line and T Cell Proliferation Assay. 7-wk-old female NOD mice were immunized subcutaneously with 100 μg of Herpes simplex virus (HSV)-2 VP16 peptide in CFA. Draining lymph nodes were removed 10 d after immunization and lymph node cells were seeded at 5 × 10⁵ per well in 96-well U-bottomed plates in serum-free media (AIM-V; GIBCO BRL) containing 5.5 × 10⁻⁵ M of 2-ME. HSV-2 VP16 peptide was added to a final concentration of 5 μM. After 3 d of culture, the medium was replaced with RPMI supplemented with 10% FCS, 5 U/ml recombinant human IL-2 (Boehringer Mannheim), 2-ME, and nonessential amino acids (Sigma Chemical Co.). T cells were grown for 6–8 d in this media and cells were fed every third day. The T cell line was maintained in culture by repeated cycles of a 3-d restimulation with irradiated NOD spleen cells and 5 μM HSV-2 VP16 peptide in AIM-V, followed by a 7-d resting period in RPMI supplemented with 10% FCS and recombinant IL-2.

T cell proliferation assays were set up in triplicates in 96-well U-bottomed plates with 2.5 × 10⁴ T cells and 2.5 × 10⁵ irradiated NOD spleen cells per well in AIM-V containing 2-ME. For blocking experiments, antibodies were added to a final concentration of 10 μg/ml. After 72 h of culture, 1 μCi of [³H]thymidine (NEN) was added to each well and incorporated radioactivity was quantitated in a beta-scintillation counter after harvesting of cells onto glass fiber filters (Wallac Oy).

Results

Expression and Purification of Soluble I-A^{g7}. Soluble I-A^{g7} was expressed in *Drosophila* Schneider cells by replacing the transmembrane and cytoplasmic segments of I-Aα and I-Aβ with leucine zipper dimerization domains from the transcription factors Fos and Jun, respectively. A short, flexible linker [Val-Asp-(Gly)₅] that encoded a SalI restriction site was placed between the extracellular domains of I-Aα and I-Aβ and the Fos/Jun segments (Fig. 1). Leucine zipper dimerization domains were previously shown to promote the assembly of HLA-DR2 and I-A^d (19, 21).

The I-Aα-Fos and I-Aβ-Jun constructs were separately cloned into the pRmHa-3 vector under the control of the

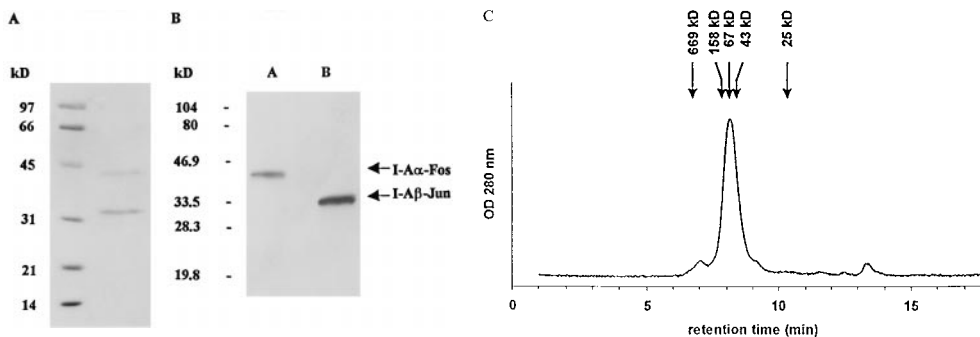


Figure 2. Biochemical characterization of soluble I-A^{g7}. I-A^{g7} was purified from concentrated supernatants by affinity chromatography using a mAb specific for the I-Aβ chain (10-2.16). (A) Purified I-A^{g7} was analyzed by SDS-PAGE (12%) and stained with Coomassie Blue. (B) The identity of α and β chains was confirmed by Western blot analysis, using polyclonal antisera raised against the Fos and Jun leucine zipper segments. The migration of

I-Aα-Fos and I-Aβ-Jun was similar to α and β chains of HLA-DR2 expressed with these leucine zipper dimerization domains (19). (C) HPLC gel filtration analysis of recombinant I-A^{g7}. 20 μg of I-A^{g7} were separated on a Bio-Gel SEC 30-XL column (300 × 7.8 mm; Bio-Rad) with PBS at a flow rate of 0.8 ml/min and detection at 280 nm. The following proteins were used as molecular mass markers: thyroglobulin (669 kD), aldolase (158 kD), BSA (67 kD), ovalbumin (43 kD), and chymotrypsinogen A (25 kD).

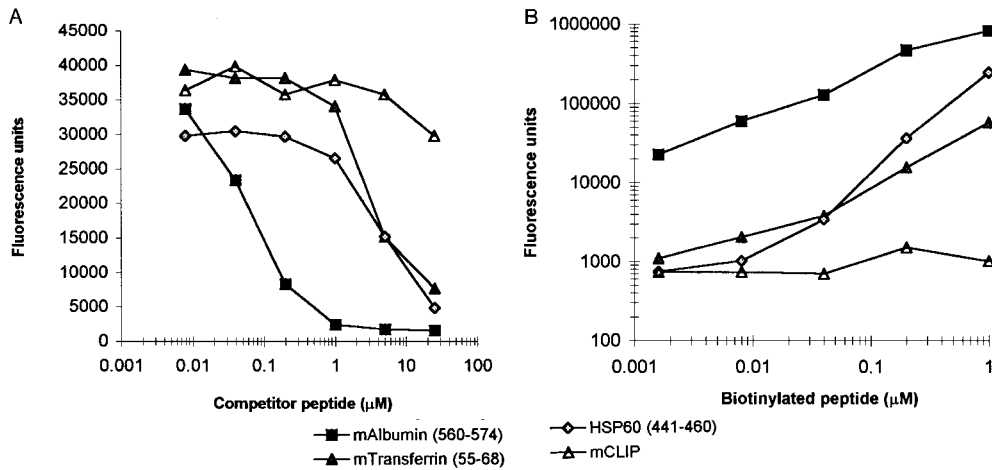


Figure 3. Binding of naturally processed peptides and peptides from islet autoantigens to soluble I-A^{g7}. Binding of peptides to soluble I-A^{g7} was examined both in a competition assay at pH 5 (A) and in a direct binding assay at pH 5 (B). (A) Unlabeled peptides were used to compete for the binding of biotinylated transferrin peptide (1 μM) to purified I-A^{g7}. (B) In the direct binding assay, biotinylated peptides (1.6 nM–1 μM) were incubated with soluble I-A^{g7} (0.6 μg) at pH 5.0. I-A^{g7}-bound biotinylated peptides were quantitated with europium-labeled streptavidin after capture with mAb 10-2.16. Fluorescence was quantitated in a time-resolved fluorometer (Delfia; Wallac Oy).

copper-inducible metallothionein promoter (18). These constructs were cotransfected with a hygromycin selection plasmid into S2 cells and transfectants were cloned by limiting dilution after initial selection with hygromycin. Expression was induced by addition of copper sulfate to the growth medium and the protein was purified from concentrated supernatants by affinity chromatography using mAb 10-2.16. The yield was ~0.7–1.6 mg/liter of culture.

SDS-PAGE of the purified protein demonstrated two bands with an apparent molecular mass of 43.3 and 33 kD (Fig. 2 A). The identity of these bands as I-A α -Fos and I-A β -Jun, respectively, was confirmed by Western blot analysis using antisera specific for the Fos and Jun leucine zippers (Fig. 2 B). The migration of the two chains was similar to that observed for α and β chains of HLA-DR2 expressed with Fos/Jun dimerization domains (19). Purified I-A^{g7} eluted from a HPLC gel filtration column with a

molecular mass appropriate for soluble I-A^{g7} (Fig. 2 C). Importantly, the protein did not form high molecular mass aggregates, indicating that it was indeed soluble in the absence of detergent.

Peptide Binding by Soluble I-A^{g7}. Soluble I-A^{g7} was used to examine the binding of naturally processed I-A^{g7} peptides as well as peptides from islet autoantigens under detergent-free conditions (Fig. 3, Table I). This is important because detergents can have a major effect on peptide binding by MHC class II molecules. For example, detergents with unbranched intermediate length hydrocarbon chains were found to promote dissociation of CLIP from HLA-DR (22). Two different binding assays were performed. In the direct binding assay, biotinylated peptides were incubated with soluble I-A^{g7} and bound biotinylated peptides were quantitated with europium-labeled streptavidin after capture of complexes with an I-A-specific antibody. In the

Table I. Peptides That Were Examined for Binding to Purified I-A^{g7}

Peptides	Reference	Sequences
Murine serum albumin (560–574)	23	KPKATAEQLKTVMD
Murine transferrin (55–68)	23	GHNYVTAIRNQEG
Human HSP60 (441–460)	41	GCALLRCIPALDSLTPANED
Human HSP60 (169–180)	42	AQVATISANGDK
Murine insulin B (9–23)	43	SHLVEALYLVCGERG
Human GAD65 (509–528)	44	I PPSLRYLEDNEERMSRLSK
Human GAD65 (247–266)	44	NMYAMMIARFKMFPEVKEKG
Human GAD65 (524–543)	44	SRLSKVAPVIKARMEYGT
Human CPH (362–381)	17	KNSLINYLEQIHRGVKGFVR
Human CPH (440–464)	17	YFSPAVGVDFELESFSEKKEEKEEL
HSV-2 VP16 (430–444)	34	EEVDMTPADALDDFD
Murine CLIP (86–100)		PVSQMRMATPLLMP

The naturally processed peptide from murine serum albumin (res. 560–574) was found to be the best binder for I-A^{g7}, both in a direct binding assay as well as in a competition assay (see references 17, 23, 34, 41–44 for peptide sequences).

competition assay, unlabeled peptides were used at different concentrations to compete for binding of a biotinylated transferrin peptide to I-A^{G7}.

A naturally processed peptide from mouse albumin (residues 560–574) was the best binder in both assays (reference 23, Fig. 3). This peptide competed for binding of the biotinylated transferrin peptide at very low peptide concentrations and also gave very high counts in the direct binding assay, even when the concentration of the biotinylated albumin peptide was <100 nM. Strong binding could also be demonstrated for another naturally processed peptide from transferrin (res. 55–68), as well as for a peptide from HSP60 (res. 441–460) (Fig. 3). The murine CLIP peptide showed little or no binding at this pH (pH 5). Several peptides that were identified as T cell epitopes of islet antigens competed for binding to I-A^{G7} only at relatively high peptide concentrations, indicating that they were relatively poor binders. This included all three peptides from GAD 65 (res. 247–266, 509–528, and 524–543) as well as HSP60 (169–180) and insulin (9–23). The HSP60 (169–180), insulin (9–23), and GAD65 (249–263) peptides were also synthesized with an NH₂-terminal biotin group and tested in the direct binding assay. These peptides gave only low counts that were close to background (data not shown). It is important to note that the majority of the peptides that have been described as T cell epitopes actually represent the human rather than the murine sequences. Since there are sequence differences in the sequences of some of these peptides, it is possible that some of the murine peptides are stronger binders. The immunodominant myelin basic protein peptide (MBP, Ac1–11) that induces experimental autoimmune encephalomyelitis in mice with the *H-2^u* haplotype is also a low affinity binder, whereas the immunodominant T cell epitope of MBP in mice with the *H-2^s* haplotype (res. 87–99) is a high affinity binder (24).

Rapid Dissociation of CLIP from I-A^{G7} at an Endosomal pH. The transferrin peptide bound to I-A^{G7} at both an acidic and a neutral pH (Fig. 4 B). The binding kinetics were characteristic for an “empty” MHC class II molecule and similar to those previously observed for soluble HLA-DR2 (Fig. 4 C; reference 19). In contrast, CLIP bound to I-A^{G7} at a neutral pH; at an acidic pH little or no binding was detected (Fig. 4 A). Analysis of human and murine CLIP peptides has demonstrated a binding mode in which two methionine residues of CLIP occupy the P1 and P9 pockets of the MHC class II peptide binding site. These residues correspond to positions 90 and 98 of mouse as well as residues 91 and 99 of human invariant chain, respectively (25–27). Since the transferrin peptide carried a negative charge at the P9 position, the corresponding residue of CLIP (methionine 98) was substituted by aspartic acid (CLIP 98D). This single amino acid substitution resulted in strong binding both at a neutral and at an acidic pH (Figs. 4 and 5). These results demonstrated that the binding characteristics of CLIP to I-A^{G7} were due to the interaction of methionine 98 with the P9 pocket of I-A^{G7}.

MHC class II molecules assemble with invariant chain at a neutral pH in the endoplasmic reticulum and are then

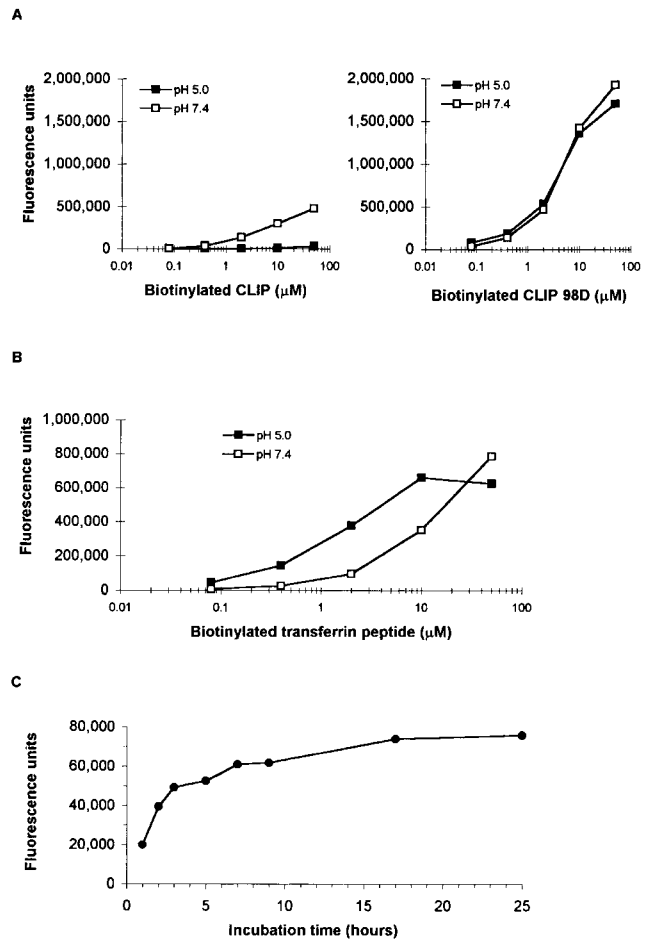


Figure 4. Binding of CLIP and transferrin (55–68) to soluble I-A^{G7}. Peptide binding to I-A^{G7} was examined using biotinylated peptides that were incubated with I-A^{G7} at 37°C. I-A-peptide complexes were captured with immobilized 10-2.16 mAb and the amount of biotinylated peptide was quantitated with europium-labeled streptavidin. (A) Poor binding of CLIP to I-A^{G7} at an endosomal pH. CLIP peptide bound to I-A^{G7} at a neutral pH, but little or no binding was detected at pH 5.0. These binding properties of CLIP were due to a poor fit of methionine 98 in the P9 pocket of I-A^{G7}, as substitution of this residue by aspartic acid (CLIP 98D) greatly enhanced binding at both pH 5 and at pH 7.4. (B) Optimal binding of transferrin peptide to I-A^{G7} at endosomal pH. The transferrin peptide bound to I-A^{G7} both at pH 5.0 and at pH 7.4. Lower peptide concentrations were required for loading of I-A^{G7} at the acidic pH. (C) Kinetics of transferrin peptide binding to I-A^{G7}. The transferrin peptide (1 μM) was incubated with I-A^{G7} (0.2 μM) at 37°C for different periods of time. Half-maximal loading was observed after ~2 h and a plateau was reached after ~16 h. The kinetics of peptide binding were therefore similar to soluble HLA-DR2 (19).

transported through the Golgi complex to an endosomal compartment that has a low pH. In this compartment, the invariant chain is proteolytically cleaved, leaving the CLIP peptide in the binding site. The removal of CLIP is catalyzed by H-2M, which allows binding of antigenic peptides (28–32). Therefore, it was important to examine the stability of complexes that were assembled at a neutral pH and then shifted to an acidic pH. For that purpose, I-A^{G7} was incubated with biotinylated CLIP at a neutral pH at 37°C. An unlabeled competitor peptide was then added in order

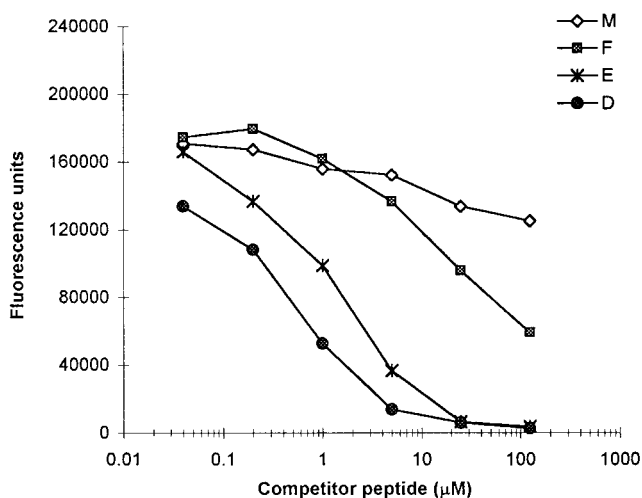


Figure 5. Substitution of CLIP at the P9 position greatly enhances binding to I-A^{g7}. Single amino acid analogues of CLIP were tested for their ability to compete for binding of the biotinylated transferrin peptide at pH 5. Although the native CLIP peptide showed little or no competition, substitution of methionine at position 98 by aspartic acid or glutamic acid greatly increased the ability of the peptide to compete for binding.

to prevent rebinding of biotinylated CLIP and the pH was changed for different periods of time (5–120 min) (Table II). After this incubation period, the sample was neutralized and the amount of bound biotinylated peptide was quantitated. This experimental design in which complexes were

formed at a neutral pH and neutralized again after incubation at pH 5 for different periods of time allowed the kinetics of CLIP dissociation to be analyzed.

These experiments demonstrated that even a brief incubation (5 min) at pH 5.0 before neutralization resulted in complete dissociation of CLIP from I-A^{g7} (Table II). Even with shorter incubation times at pH 5 (<2 min), no bound CLIP was detected (data not shown). When the complex was kept at pH 7.4, dissociation was considerably slower and bound CLIP was still detected after 120 min. The rapid, pH-dependent dissociation of CLIP was due to methionine 98 of CLIP, which occupies the P9 pocket of MHC class II molecules (25). Substitution by aspartic acid (CLIP 98D) greatly increased the stability of the complex (Table II). These results indicated that the stability of I-A^{g7}-CLIP complexes was pH dependent, and that the P9 pocket of I-A^{g7} was critical in determining this property.

A possible explanation for the pH-dependent dissociation of CLIP was that the interaction of peptide side chains with the P9 pocket was pH dependent. Therefore, a panel of CLIP analogue peptides in which methionine 98 was substituted by all naturally occurring amino acids was examined in the competition assay (Table III). These experiments demonstrated three important properties of I-A^{g7}: (i) At both an acidic and a neutral pH, I-A^{g7} has a strong preference for a negatively charged residue at P9. (ii) The interaction of I-A^{g7} and CLIP is relatively weak at a neutral pH, and there is little or no interaction at an acidic pH.

Table II. Rapid Dissociation of CLIP from I-A^{g7} at an Endosomal pH

Conditions	CLIP		CLIP 98D	
	pH 7.4	pH 5.0	pH 7.4	pH 5.0
	<i>Fluorescence U</i>			
I-A ^{g7} , no biotinylated peptide	462		636	
Biotinylated CLIP or CLIP 98D*	58762		670032	
Biotinylated CLIP or CLIP 98D plus competitor [†]	12855		775282	
Additional incubation [§]				
5 min	11190	712	750899	419044
10 min	10355	798	667831	425805
20 min	10244	813	739503	303485
30 min	8237	680	740163	342409
60 min	6783	587	788291	254762
120 min	3948	515	758073	206928

Dissociation of CLIP was examined at pH 5.0 and pH 7.4. The biotinylated CLIP peptide was first bound to I-A^{g7} at a neutral pH by incubation at 37°C. The complex was then shifted to pH 5.0 for 5–120 min at 37°C and an excess of unlabelled competitor peptide was added to prevent rebinding of biotinylated CLIP. The sample was then neutralized and the amount of bound biotinylated CLIP peptide was determined as described above. Even a brief incubation at pH 5.0 resulted in complete dissociation of CLIP from I-A^{g7}. At pH 7.4, dissociation was significantly slower and bound CLIP was still detectable after 2 h of incubation. Substitution of methionine 98 by aspartic acid (CLIP 98D) greatly enhanced binding, as well as the stability of the complex at a neutral or an acidic pH.

* 16 h incubation at 37°C.

[†] 16 h incubation at 37°C, followed by an addition of unlabeled competitor peptide.

[§] Additional incubation at 37°C at the indicated pH in the presence of competitor peptide, followed by neutralization of the pH 5 samples. Length of incubation is given in min.

Table III. *pH-dependent Interaction of Different Peptide Side Chains with the P9 Pocket of I-A^{g7}*

Amino acid at position P9 of CLIP	IC ₅₀	
	pH 5.0	pH 7.4
	<i>μM</i>	
M	>125	50
P	>125	>125
G	>125	10
A	15	2.0
V	17	50
L	>125	40
I	>125	>125
F	50	80
Y	60	>125
W	>125	80
H	>125	4.0
K	>125	>125
R	>125	>125
D	0.5	0.5
E	1.6	0.4
Q	>125	25
N	125	80
S	20	40
T	40	>125
C	20	40

Residue 98 of CLIP was substituted by all naturally occurring amino acids. These analogues were examined in the competition assay, at both an acidic and neutral pH. Peptides with a negative charge at P9 competed for binding both at pH 5.0 and pH 7.4 at low peptide concentrations. In contrast, binding of other analogues was strongly pH dependent. For example, the histidine analogue did not compete for binding at pH 5.0, but competed well at a neutral pH.

(iii) The interaction of some peptide side chains with the P9 pocket is pH dependent. Particularly striking is the observation that a CLIP analogue with histidine at P9 competes well at pH 7.4 (IC₅₀ of 4 μM), but not at pH 5.0 (IC₅₀ of >125 μM).

I-A^{g7} Forms Long-lived Complexes with Naturally Processed Peptides, But Not SDS-resistant Dimers. Since it has been hypothesized that the interaction of peptides with I-A^{g7} is short lived (17), we determined the dissociation rates of several peptides, which included the naturally processed transferrin and serum albumin peptides, the CLIP 98D analogue, and peptides that have been identified as T cell epitopes in NOD mice (Fig. 6, Table IV). Purified I-A^{g7} was first incubated with one of the biotinylated peptides (1 μM), then the pH was adjusted to pH 7.4 or maintained at pH 5.0 and mouse serum albumin peptide was added to a final concentration of 100 μM to prevent rebinding of dissociated biotinylated peptide. The GAD 65 peptide dissociated very rapidly and a t_{1/2} value could not be estimated. In

contrast, complexes of I-A^{g7} and several other peptides were long lived, particularly at a neutral pH. The estimated t_{1/2} value for the serum albumin peptide was >72 h and for the transferrin peptide ~45 h at pH 5.0 and >72 h at a neutral pH. The dissociation of the CLIP 98D analogue was faster, with a t_{1/2} value of ~23 h at pH 5.0 and ~70 h at pH 7.4 (Fig. 6). These results demonstrated that I-A^{g7} forms long-lived complexes with naturally processed peptides and that the dissociation rate of peptides was affected by pH.

Nevertheless, I-A^{g7}-peptide complexes were not resistant to SDS (Fig. 7). I-A^{g7} (0.6 μg) was loaded at pH 5.0 with four different peptides (transferrin, serum albumin, CLIP, or CLIP 98D analogue) by overnight incubation at 37°C. Fractions of I-A^{g7}-peptide complexes (0.1 μg) were examined for the formation of SDS-resistant dimers by Western blot analysis using polyclonal antisera specific for the Fos and Jun segments. An SDS-resistant dimer could not be detected with any of the peptides tested. Therefore, the SDS assay does not determine whether peptides were bound to I-A^{g7} before addition of detergent. Soluble HLA-DR1 expressed in insect cells forms SDS-stable dimers with the influenza hemagglutinin (307–319) peptide (33). This indicates that SDS-sensitivity is not a general property of soluble MHC class II molecules expressed in insect cells.

Since several of the peptides that were identified as T cell epitopes of islet autoantigens were poor binders, we examined if peptides with such binding properties could induce a T cell response. We chose the peptide from HSV-2 VP16, which has been reported to bind to HLA-DQ8 (34), since it represented a foreign antigen. This peptide showed little binding in the direct binding assay, but competed for binding of the biotinylated transferrin peptide at high peptide concentrations (data not shown). NOD mice were primed with this peptide in CFA and a T cell line specific for the HSV-2 peptide could be established from draining lymph nodes by restimulation with the HSV-2 peptide (Fig. 8 A). This T cell line was I-A^{g7} restricted since the response could be completely blocked by mAb 10-2.16, but not by a control antibody (Y3-P) (Fig. 8 B). Stimulation of this T cell line required the continuous presence of the peptide in the culture. Even a single wash of the APCs after overnight incubation with the peptide completely eliminated the T cell response (Fig. 8 C, lane 2). These results demonstrate that T cells from NOD mice can be primed even by a peptide that is a poor binder for I-A^{g7}.

Discussion

Soluble I-A^{g7} was used to define the biochemical requirements for peptide loading by this MHC class II molecule, which confers susceptibility to type I diabetes in NOD mice. Several peptides, including two naturally processed peptides from exogenous antigens, bound to I-A^{g7}. The interaction with the invariant chain-derived CLIP peptide was relatively weak and pH dependent. CLIP bound to I-A^{g7} at a neutral pH, but rapidly dissociated at an endosomal pH. This prop-

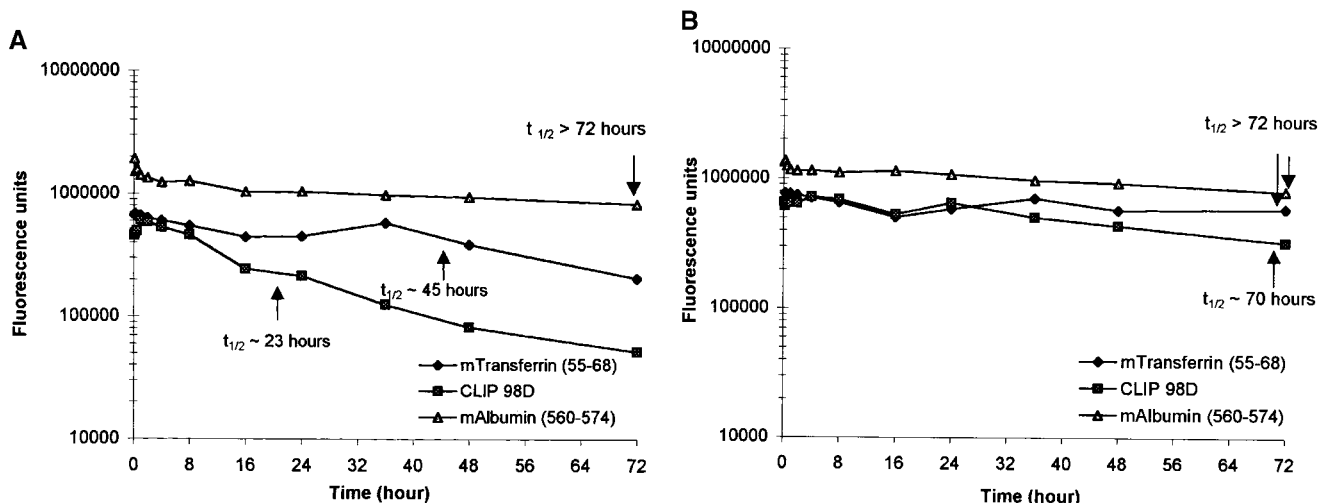


Figure 6. pH-dependent dissociation of peptides from I-A^{g7}. I-A^{g7} (0.6 μg) was incubated with biotinylated peptides from mouse albumin and transferrin or the CLIP 98D analogue (all at 1 μM) at 37°C for 18 h at pH 5.0. The pH of the reaction was either kept at pH 5.0 (A) or adjusted to pH 7.4 (B). At this time, an excess of mouse serum albumin peptide (100 μM) was added as a competitor to prevent rebinding of biotinylated peptide. After incubation at the indicated pH for 5 min to 72 h, bound biotinylated peptide was quantitated as described in Materials and Methods. All three peptides dissociated slowly at a neutral pH, but dissociation of the transferrin and CLIP 98D analogue was more rapid at pH 5.0.

erty of CLIP was due to methionine 98, which occupies the P9 pocket of the MHC class II peptide binding site (25). Substitution of this residue of CLIP greatly increased the stability of the complex, both at an acidic and neutral pH. These characteristics of I-A^{g7} result in the rapid generation of empty molecules at an endosomal pH.

Several mutant cell lines have been described that are defective in antigen presentation due to a lack of DM expression. In these cell lines, MHC class II-CLIP complexes accumulate because DM does not catalyze the re-

moval of CLIP (32). For several human and murine MHC class II molecules, CLIP peptides are abundant among naturally processed peptides in normal cell lines, reflecting a relatively stable interaction (35, 36). For example, CLIP peptides have been eluted from purified I-A^b and I-A^d. CLIP was found to bind with a relatively high affinity to I-A^d (IC₅₀ of 14 nM) and was one of the best binders identified for this class II molecule (36). These results are important, since I-A^{g7} and I-A^d share the same I-A α chain (12). Thus, the binding properties of CLIP to I-A^{g7} appear to be due to the unique I-A^{g7} β chain.

The requirement for DM in the presentation of antigens by I-A^{g7} has been analyzed using T cell hybridomas specific for chicken ovalbumin (37). For all T cell hybridomas, the antigen was presented by transfectants that expressed only I-A^{g7} and invariant chain, but not H-2M, indicating that

Table IV. I-A^{g7} Forms Long-lived Complexes with Certain Peptides

Peptide	Dissociation time (t _{1/2})	
	pH 5.0	pH 7.4
Murine serum albumin (560-574)	>72 h	>72 h
Murine transferrin (55-68)	~45 h	>72 h
Human HSP60 (441-460)	~14 h	~25 h
Human GAD65 (249-263)	not detectable	not detectable
HSV-2 VP16 (430-444)	not detectable	not detectable
Murine CLIP	not detectable	~1 h
Murine CLIP 98D analogue	~23 h	~70 h

The dissociation time (t_{1/2}) of a panel of peptides was examined as described in Fig. 6. Several peptides formed long-lived complexes with I-A^{g7}, particularly at a neutral pH. The peptides included the naturally processed peptides from serum albumin and transferrin, as well as a peptide from human HSP60 (res. 441-460) that has been identified as a T cell epitope in NOD mice. The dissociation of the CLIP peptide at pH 5.0 was very rapid and a half-life could not be determined. These data demonstrate that I-A^{g7}-peptide complexes can be long lived, even though I-A^{g7}-peptide complexes are not SDS resistant.

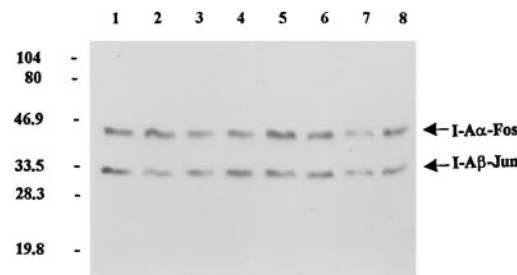


Figure 7. I-A^{g7}/peptide complexes are not SDS-resistant. I-A^{g7} (0.6 μg) was loaded at pH 5.0 with four different peptides (transferrin, mouse serum albumin, CLIP, or CLIP 98D, each at 5 μM) by overnight incubation at 37°C. Fractions of I-A^{g7}-peptide complexes (0.1 μg) were examined for the formation of SDS-stable dimers by Western blot analysis using polyclonal antisera specific for the Fos and Jun segments. Samples were either not boiled (lanes 1-4) or boiled (lanes 5-8). Lanes 1 and 5, transferrin peptide; lanes 2 and 6, mouse serum albumin peptide; lanes 3 and 7, CLIP peptide; lanes 4 and 8, CLIP 98D analogue.

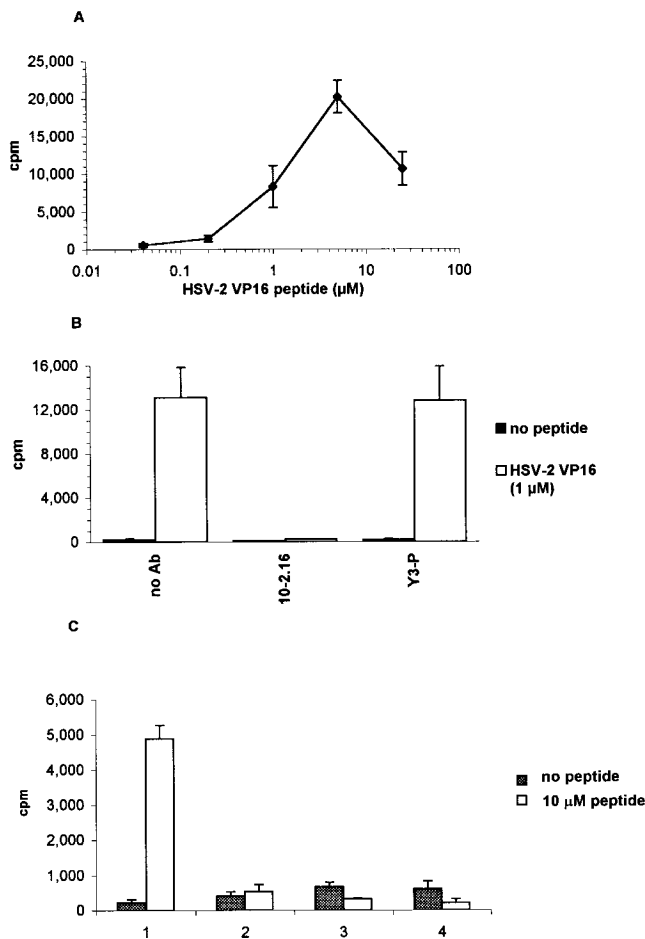


Figure 8. NOD mice can be primed by a foreign peptide that is a weak binder to I-A^{g7}. A T cell line specific for HSV-2 VP16 (res. 430–444) was generated by immunizing NOD mice with this peptide in CFA. (A) Dose–response of the T cell line to the HSV-2 peptide using irradiated NOD spleen cells as APCs. T cell proliferation was determined by [³H]thymidine incorporation. (B) The HSV-2–specific T cell line was I-A^{g7} restricted since proliferation was blocked by mAb 10-2.16 but not by a control antibody (Y3-P). (C) Stimulation of HSV-2–specific T cells required continuous presence of the peptide. Splenocytes from NOD mice were pulsed overnight ± 10 μM HSV-2 peptide in serum-free media (AIM-V) and then split into four aliquots: 1, No wash of APCs before coculture with T cells; 2, APCs washed once with 10 ml of RPMI; 3, APCs washed twice and incubated for 30 min between washes; and 4, APCs washed twice and incubated for 60 min between washes.

the murine DM homologue is not essential for antigen presentation by I-A^{g7}. For two out of four hybridomas with a distinct epitope specificity, presentation was enhanced by coexpression of H2-M. These results indicate that presentation of certain epitopes by I-A^{g7} can be enhanced by H-2M. This may be due to “editing” of peptide content as well as stabilization of empty I-A^{g7} molecules by H-2M, as described for human MHC class II molecules (30, 31, 38).

The recently solved crystal structure of I-A^d demonstrates several features of I-A–peptide interactions that are important in this context (16). Detailed analysis of the I-A^d peptide binding motif demonstrated that only two peptide residues in a 6-amino acid core were important for binding (39). These residues are located in the P4 and P9 pockets of

the I-A^d crystal structure. I-A^d and I-A^{g7} have the same α chain sequence, but differ at 17 positions of the β chain. An important difference is the presence of an interchain salt bridge between β57 Asp and α76 Arg in I-A^d, which is absent in I-A^{g7} (β57 Ser). In I-A^{g7}–peptide complexes, a salt bridge may instead be formed by P9 Asp or P9 Glu of bound peptides and α76 Arg. This would explain the strong preference for negatively charged residues at the P9 position, which was first noted among naturally processed peptides eluted from I-A^{g7} (23). In contrast, I-A^d has a preference for alanine or serine at the P9 position. An I-A^{g7} peptide binding motif has been proposed, in which a preference for aliphatic residues was noted at P6 and for hydrophobic residues at P9 (40). Also, at P11 binding was enhanced by the presence of aspartic acid. It appears that these data are in general agreement with other data on I-A^{g7}–peptide interactions, provided that the relative positions are reassigned such that these data reflect the preferences of the P4, P7, and P9 pockets of I-A^{g7}.

Are I-A^{g7} molecules poor peptide binders or are only some of the peptides that have been identified thus far poor ligands? This may be a separate issue from the lack of SDS resistance. The analysis of soluble I-A^{g7} clearly demonstrates that this MHC class II molecule binds peptides. Two naturally processed peptides from extracellular antigens, mouse albumin and transferrin, are strong binders, indicating that I-A^{g7} does not have a global defect in presentation of exogenous antigens. Peptide binding by purified I-A^{g7} has only been reported in two studies that used detergent-solubilized molecules. In one of these studies, little or no specific peptide binding could be demonstrated (17). In the other study, high detergent concentrations (2% NP-40) were used in the peptide binding assay (40). Binding was demonstrated for a hen egg lysozyme peptide (res. 9–27), but not for the mouse albumin peptide that had been eluted from purified I-A^{g7} and that represents the best binder for soluble I-A^{g7} (23). It is possible that these difficulties in examining peptide binding by I-A^{g7} are related to the isolation method, in particular to the use of certain detergents. The naturally processed transferrin and serum albumin peptides formed long-lived complexes with I-A^{g7}, in particular at a neutral pH. However, several peptides that have been identified as T cell epitopes of islet antigens were found to be poor binders. A similar observation has been made in the experimental autoimmune encephalomyelitis model, since the immunodominant MBP peptide (Ac1-11) in mice with the H-2^u haplotype is a very weak binder (24). Taken together, our data indicate that some peptides form long-lived complexes with I-A^{g7}. However, it is possible that a smaller fraction of I-A^{g7} molecules represent long-lived class II–peptide complexes and/or that the average affinity of I-A^{g7} bound peptides is lower than that of peptides bound to other I-A molecules.

I-A^{g7} immunoprecipitated from splenocytes of NOD mice does not form SDS-resistant dimers (17). This property was also observed for soluble I-A^{g7}, even when the molecules were loaded with peptides. Therefore, it appears that SDS-sensitivity of the dimer is an intrinsic property of

I-A^{g7} and does not directly reflect peptide content. SDS sensitivity of the I-A^{g7} dimer could be due to polymorphic residues at the dimerization interface of I-A α and I-A β . Mutational analysis of I-A^{g7} indicated that β 56 and β 57 do not determine SDS-sensitivity (17). The absence of the salt bridge between β 57 and α 76 is therefore not responsible for the lack of SDS resistance. It is also important to keep in mind that instability of the dimer in SDS does not indicate

that the dimer is also unstable under native conditions. In fact, analysis of I-A^{g7} purified from B cells did not indicate that the dimer was unstable in the absence of SDS (17).

The pH-dependent interaction of I-A^{g7} results in the rapid generation of empty molecules at an endosomal pH. These results demonstrate a novel mechanism by which the presentation of antigenic peptides can be affected by a disease-associated MHC class II molecule.

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