T Cell Recognition of Major Histocompatibility Complex Class II Complexes with Invariant Chain Processing Intermediates

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Summary

Peptides from the lumenal portion of invariant chain (Ii) spanning residues 80–106 (class II–associated Ii peptide [CLIP]) are found in association with several mouse and human major histocompatibility complex (MHC) class II allelic variants in wild-type and presentation-deficient mutant cells. The ready detection of these complexes suggests that such an intermediate is essential to the MHC class II processing pathway. In this study, we demonstrate that T cells recognize CLIP/MHC class II complexes on the surface of normal and mutant cells in a manner indistinguishable from that of nominal antigenic peptides. Surprisingly, T cell hybrids specific for human CLIP bound to murine MHC class II molecule I-A^b and a new monoclonal antibody 30-2 with the same specificity, recognize two independent epitopes expressed on this peptide/class II complex. T cell recognition is dependent on a Gln residue (position 100) in CLIP, whereas the 30-2 antibody recognizes a Lys residue at position 90. These two residues flank the 91-99 sequence that is conserved among human, mouse, and rat Ii, potentially representing an MHC class II–binding site. Our results suggest that the COOH-terminal portion of CLIP that includes TCR contact residue Gln 100 binds in the groove of I-A^b molecule. Moreover, both T cells and the antibody recognize I-A^b complexed with larger Ii processing intermediates such as the ~12-kD small leupeptin-induced protein (SLIP) fragments. Thus, SLIP fragments contain a CLIP region bound to MHC class II molecule in a conformation identical to that of a free CLIP peptide. Finally, our data suggest that SLIP/MHC class II complexes are precursors of CLIP/MHC class II complexes.

Invariant chain (Ii)[1] is a type II transmembrane glycoprotein transiently associated with MHC class II molecules during their intracellular assembly and transport (1). The association of class II with Ii interferes with peptide binding to the peptide-binding groove of MHC class II molecules (2–4). Large multimeric complexes of three Ii polypeptides and three MHC class II $\alpha\beta$ dimers form rapidly in the endoplasmic reticulum (ER) (5). These complexes are transported across Golgi stacks to the *trans*-Golgi network and are sorted to the endosomal compartment (1). Several studies in mice with a targeted disruption of the Ii gene demonstrated that this sorting event is to a large extent mediated by Ii (6–8). Earlier in vitro studies revealed an endosomal targeting signal in the first 16 amino acid residues in the cytoplasmic tail of Ii (9). After arrival in endosomes, Ii is cleaved by endosomal proteases. Studies using protease inhibitors indicated that an aspartyl proteinase(s) and a cysteine proteinase(s) are sequentially involved in endosomal degradation of Ii in B cells (10, 11). In cells treated with a cysteine proteinase inhibitor, leupeptin, two major intermediates of Ii processing were demonstrated (10, 11). These intermediates are NH_2-terminal 21–24-kD fragments of Ii (leupeptin-induced protein [LIP]) and 11–14-kD fragments (small leupeptin-induced protein [SLIP]).

Sequence analysis of peptides associated with several allelic variants of human and mouse MHC class II in normal cells demonstrated an abundant set of peptides with ragged NH_2- and COOH-termini derived from Ii residues 80–106 (12–15). Importantly, these peptides are associated with the majority of MHC class II molecules expressed in human presentation deficient mutant cell lines. The peptides were

[1] *Abbreviations used in this paper:* CLIP, class II–associated invariant chain peptide; ER, endoplasmic reticulum; HEL, hen egg lysozyme; HPLC, high performance liquid chromatography; Ii, invariant chain; SLIP, small leupeptin-induced protein; TfR, transferrin receptor.

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named CLIP (class II–associated Ii peptide; 16). Recent studies using a series of deletions in the lumenal portion of Ii indicate that the same 80–110 region of Ii is essential for its binding to MHC class II (17–19). Altogether, these results suggest that CLIP/MHC class II complexes are essential intermediates in the MHC class II processing pathway. The relationship between CLIP and larger Ii fragments such as LIP and SLIP, however, remains unclear. Thus, it is not known whether CLIP represents a remnant of Ii still associated with MHC class II after Ii truncation (12) or, alternatively, if CLIP is first released during Ii proteolysis and subsequently rebinds in the peptide-binding groove (12, 16, 20).

To address these questions, we have generated and characterized a panel of T cell hybrids recognizing human CLIP peptides bound to mouse MHC class II molecule I-A^b. Using these T cells and a new mAb, 30-2, which recognizes the same complex (Eastman, S., M. Deftos, P. DeRoos, D. H. Hsu, L. Teyton, N. S. Braunstein, C. J. Hackett, and A. Yu. Rudensky, manuscript submitted for publication), we show here that the CLIP region of Ii binds to MHC class II molecule in a conformation similar to that of the free peptide. Mapping of antibody and T cell epitopes within the CLIP peptide allowed us to identify the conserved 91-99 region of Ii as a peptide capable of competitively inhibiting MHC class II binding to antigenic peptides. Further, we demonstrate that the ~12-kD SLIP fragment of Ii is a precursor of CLIP. Our data suggest a model of CLIP binding to I-A^b with the COOH terminus of this peptide bound in the MHC class II–binding groove.

Materials and Methods

Mice. C57BL/6, BALB/c, and B6.C-H-2^{bm12} mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antibodies. The mAb 30-2 recognizing human CLIP/I-A^b complex was generated in our laboratory (Eastman, S., et al., manuscript submitted for publication), and the the YA*e* antibody recognizing complex of I-A^b molecule with Eα52-68 peptide was characterized earlier (21). The mAbs PIN.1 and 1G12, which recognize the cytoplasmic tail of the human Ii chain (5) and human transferrin receptor (TfR), respectively, were kindly provided by Dr. Janice Blum (University of Indiana, Indianapolis, IN). Antibody BU45 against lumenal portion of human Ii chain was purchased from The Binding Site (San Diego, CA). Mouse hybridoma HP6035 producing mAb against human Ig κ chain was purchased from American Type Culture Collection (Rockville, MD). Antibody Y237, which recognizes I-A^b, was kindly provided by Dr. Donal Murphy (New York State Department of Health, Albany, NY). Hybridomas secreting I-A^b–specific mAbs Y3P, AF6-120.1.2, and M5/114 are maintained in our laboratory. All antibodies were purified from culture supernatants or ascitic fluids on protein G–Sepharose columns.

Cell Lines. The mouse B cell lymphoma LB27.4 (I-$A^{b\times d}$) was cotransfected with a genomic construct of the human Ii (gift from Dr. Paul Roche, National Institutes of Health, Bethesda, MD) and a vector carrying the neomycin resistance pMC1pA. Transfectants were selected for Ii expression by Western blotting of cell lysates using the anti–human Ii antibody PIN.1. The T2 cell line and T2 transfected with I-A^k (22) were provided by Dr. Peter Cresswell (Yale University, New Haven, CT). T2 cells transfected with I-A^b were characterized earlier (Eastman, S., et al., manuscript submitted for publication). The human lymphoblastoid B cell line Sweig was transfected with I-A^b α and β cDNAs cloned into expression vectors pNA and pHA carrying neomycin and hygromycin resistance genes, respectively, and was screened for surface expression of I-A^b using Y3P and AF6-120.1.2 antibodies. T cell hybridomas CLIP 51.5, CLIP 51.10, CLIP 51.11, CLIP 51.24, CLIP 51.26, CLIP 51.31, CLIP 51.38, and CLIP 51.40 were generated by fusion of BW5147 $\alpha^-\beta^-$ thymoma with activated lymph node T cells from H-2^b mice immunized with human CLIP (Ii81-104) peptide in CFA. Alloreactive T cell hybridoma BPB211 specific for I-A^b was generated by fusion of BW5147 cells with activated lymph node cells derived from a B10.Br (H-2^k) mouse immunized with cells expressing I-A^b.

All cell lines have been maintained in RPMI1640 supplemented with 5% FCS, L-glutamine, penicillin/streptomycin (all from GIBCO BRL, Gaithersburg, MD) in a 5% CO_2 atmosphere at 37°C.

Peptides. Peptides were synthesized using an automated peptide synthesizer (Synergy 432: Applied Biosystems, Inc., Foster City, CA) using F-moc chemistry. Peptides were analyzed by reverse-phase HPLC. Purity of the peptides used in this study was greater than 90%.

T Cell Assays. 5×10^4 T cell hybridomas were cocultured with 5×10^4 APCs per well in 200 μl of supplemented RPMI in 96-well flat-bottomed tissue culture microplates at 37°C in the presence of titrated amounts of peptides. After 18 h of culture, 50-μl aliquots of culture supernatants were tested for IL-2 using the IL-2–sensitive indicator cell line HT2 (5×10^3 cells per well). After 20 h of culture, 10 μl of Alamar Blue solution (Alamar Biosciences, Sacramento, CA) was added to the wells, color reaction developed for 18 h at 37°C, and OD ($A_{570/600}$) was measured using an ELISA plate reader (EL311; Bio-Tek Instruments, Winooski, VT). To assay T cell response to MHC class II molecules captured by anti-MHC antibodies, ELISA microplates (Immulon 4; Dynatech, Chantilly, VA) were coated overnight at 4°C with 30-2, anti–I-A^b, or control antibodies (5 μg/ml in PBS, pH 8.0, 50 μl per well). Since PIN.1 mAb loses its activity during purification, in experiments with the lysates of leupeptin treated cells, plates were coated with goat anti–mouse IgG-Fc antibodies (Accurate Chemical and Scientific Corp., Westbury, NY) followed by PIN.1 antibody, anti I-A^b, or control antibodies containing culture supernatants. The plates were then blocked with PBS containing 1% BSA and 0.4% NP-40 (Sigma), washed three times with PBS, 0.1% NP-40. Cell lysates (50 μl per well) were added and incubated overnight at 4°C. The wells were subsequently washed with PBS, 0.1% NP-40 with PBS, 1% BSA, and finally with RPMI, 5% FCS, three times each. T cells were added to the plate, and overnight culture supernatants were tested using HT2 cells.

Cell Lysates, Western Blotting, and MHC Class II ELISA. Cells were harvested in the logarithmic phase of growth, washed twice with PBS, and lysed at 10^7 cells per ml in PBS containing 1% NP-40, 5 mM EDTA, 0.1 mM PMSF, 0.2 mM TLCK, 10 U/ml aprotinin for 20 min on ice. Lysates were cleared by centrifugation at 16,000 *g* at 4°C. To enrich for SLIP fragments in some experiments, 2.5×10^7 T2-I-A^b cells were cultured in the presence of 100 μg/ml leupeptin for 24 h, lysed as described above, and treated with 200 μl of mAb BU45-coated protein A–Sepharose (Pharmacia, Uppsala, Sweden) (200 μl of ascites per 1.2 ml of 50% gel slurry) for 1 h in 4°C. The Sepharose beads were removed by centrifugation, supernatants were collected, and the procedure was repeated five times followed by a 2-h incubation with protein A–Sepharose. SDS-PAGE and Western blot analysis

was performed as described before (21). Briefly, 50, 10, and 2 μl of the lysates were run over 10–20% polyacrylamide gradient gels and transferred onto nitrocellulose filters. Ii and its fragments containing intact cytoplasmic tail were detected with PIN.1 antibody. The MHC class II sandwich ELISA was previously described (Eastman, S., et al., manuscript submitted for publication).

Peptide-binding Assay. Peptide binding to I-A^b on the live B cells was performed as described before (Eastman, S., et al., manuscript submitted for publication). Briefly, splenocytes from C57BL/6 mice were depleted of T cells by treatment with anti–Thy-1 antibody Y19 and complement. Live B cells were isolated by centrifugation over a Ficoll density gradient. 3–4 × 10^5 B cells were preincubated with or without inhibitor peptides for 1 h at 37°C, 5% CO_2, in 50 μl of supplemented RPMI in the wells of round-bottomed 96-well plates. After this first incubation, reference peptide Eα52-68 was added to each well in 20 μl of supplemented RPMI at final concentrations of 6, 20, and 60 μM, and was incubated for 4 h as described above. Cells were stained with YAe antibody and analyzed on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Biosynthetic Labeling and Immunoprecipitation. Pulse-chase labeling of cells with [^{35}S]methionine/cysteine (Tran^{35}S-Label; ICN, Costa Mesa, CA) in the presence of 100 μg/ml leupeptin and immunoprecipitation were performed as previously described (Eastman, S., et al., manuscript submitted for publication). Immunoprecipitates were analyzed by Tris-tricine SDS-PAGE as described previously (23).

Results

CLIP/I-A^b–specific T Cells and mAb Recognize Different Epitopes. To define interactions of class II with Ii peptide, a panel of T cell hybridomas was generated by immunization of H-2^b mice with human CLIP peptide and subsequent fusion of activated lymph node cells with the BW5147 fusion partner. Six hybridomas were selected for their response to synthetic CLIP peptide–pulsed C57BL/6 splenocytes and cloned by limiting dilution. All of the hybridomas responded to CLIP presented by C57BL/6 (I-A^b), but not by either BALB/c (I-A^d) or bm12 (I-A^{bm12}) splenocytes (Fig. 1 *a*). Control I-A^b binding HEL74-88 or Eα52-68 peptides as well as mouse Ii81-104 peptide did not induce any response in these T cells (data not shown). Experiments using mouse B cell lines transfected with human Ii and human B cells transfected with I-A^b demonstrated that CLIP-specific T cells recognize naturally occurring CLIP/I-A^b complexes on the surface of wild-type and antigen processing–deficient cells, as shown in Fig. 1 *b*. Nontransfected T2 cells did not activate T cells, while T2 cells expressing I-A^b molecule did. Similarly, human B cell line Sweig was able to stimulate T cell hybridomas only after transfection with I-A^b genes. Finally, T cell hybridomas did not respond to mouse B cell lymphoma LB27.4. However, these cells transfected with human Ii gene (LB27.4huIi) induced strong T cell response (Fig. 1 *b*).

To compare the specificity of T cell hybrids with that of previously described CLIP/I-A^b mAb, 30-2 (Eastman, S., et al., manuscript submitted for publication) we performed antibody-blocking experiments. Surprisingly, T cell response to CLIP was not blocked by the 30-2 antibody. In contrast, some but not all I-A^b–specific antibodies inhibited T cell response (Fig. 2, data not shown). In controls, differ-

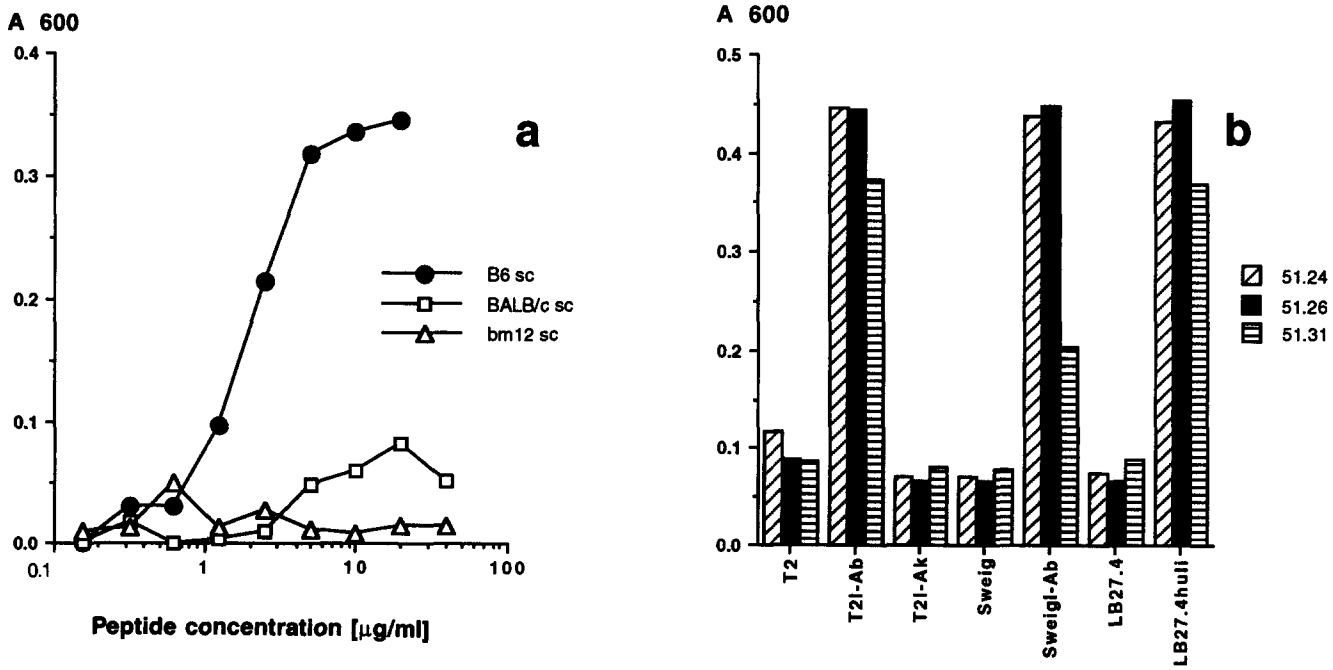


Figure 1. CLIP-specific T cells respond to synthetic and naturally processed CLIP peptide bound to I-A^b. (*a*) C57BL/6 (I-A^b), B6.C-H-2^{bm12} (I-A^{bm12}), and BALB/c (I-A^d) splenocytes pulsed with titrated amounts of synthetic CLIP (Ii81-104) peptide stimulate T cell hybridoma 51.24. (*b*) CLIP-specific 51.24, 51.26, and 51.31 T cell hybrids respond to naturally processed CLIP presented by human T2 and Sweig cell lines transfected with I-A^b or by the mouse I-A^b–positive LB27.4 B cell line transfected with human Ii. Corresponding nontransfected cell lines were used as controls. T cell activation was measured by the IL-2–dependent HT-2 cell line using Alamar blue colorimetric assay.

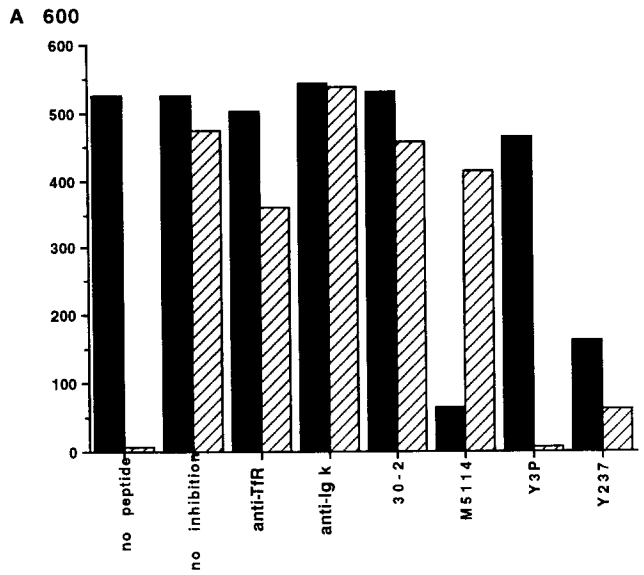


Figure 2. Inhibition of CLIP-specific T cell response by some, but not all, anti-I-A^b mAbs. LB27.4 cells were incubated with Ii81-104 peptide (1 μg/ml) and antibodies (10 μg/ml) and CLIP-specific hybridoma 51.31 (*hatched bars*). The alloreactive I-A^b specific–hybridoma BPB211 was used as a control (*filled bars*). Background response of BPB211 hybridoma to syngeneic (H-2^k) spleen cells was <OD 0.10 (represented by 100 on the scale). T cell response was measured as described above.

Table 1. *Sequences of Synthetic Ii Peptides*

	81 85 90 95 100 104
Ii81-104	LPKPPKPVSKMRMATPLLMQALPM
	** * ****
Ii81-99	LPKPPKPVSKMRMATPLLM
Ii81-93	LPKPPKPVSKMRM
Ii81-91	LPKPPKPVSKM
Ii85-104	PKPVSKMRMATPLLMQALPM
Ii91-104	MRMATPLLMQALPM
Ii93-104	MATPLLMQALPM
Ii89-101	SKMRMATPLLMQA
Ii85-99	KPVSKMRMATPLLM
Ii91-99	MRMATPLLM
Ii91-104(100R)	MRMATPLLMRALPM
Ii91-104(101P)	MRMATPLLMQPLPM
Ii91-104(102M)	MRMATPLLMQAMPM
Ii91-104(103S)	MRMATPLLMQALSM
Ii81-104 (mouse sequence)	LPKSAKPVSQMRMATPLLMRPMSM

*Residues that are different in mouse and human CLIP sequences

ential inhibition of response of I-A^b–specific alloreactive hybrid by this panel of antibodies was demonstrated.

Fine Specificity of CLIP/I-A^b Recognizing T Cells. To map the T cell recognition site within the CLIP sequence, we synthesized a series of truncation variants of the CLIP peptide (Table 1) and analyzed their ability to induce T cell response and to bind to the I-A^b molecule. COOH-terminal truncation variants lacking residues 100-104 failed to stimulate T cell hybridomas (Fig. 3 *a*, peptides Ii81-99, Ii81-93, and Ii81-91). In contrast, Ii85-104 peptide with the NH_2-terminal truncation stimulated T cells as strongly as the full-length CLIP peptide (Ii81-104). This suggests, that the T cell recognition site(s) is contained within COOH-terminal portion of the CLIP peptide. Since sequences of mouse and human Ii are identical between residues 91 and 99, we suggest that T cell specificity is confined to four residues, 100–103, at which mouse and human Ii sequences differ. We generated four peptide variants with single amino acid substitutions changing human Ii residues for corresponding mouse residues. Two of these peptides with 102 Leu → Met and 103 Pro →Ser mutations induced response of T cells similar to that of the wild-type Ii91-104 peptide (Fig. 3 *b*, peptides Ii91-104, Ii91-104102M] and Ii91-104[103S]). Proline substitution for alanine at position 101 (101 Ala → Pro) decreased T cell response significantly. The substitution of glutamine at position 100 with arginine (100 Gln → Arg), however, abolished the response completely (Fig. 3 *b*, peptides Ii91-104, Ii91-104[101P] and Ii91-104[100R]), indicating that Gln 100 is critical for T cell recognition. Furthermore, truncated peptide Ii89-101 was able to activate T cells similarly to peptide Ii91-104 (Fig. 3 *a*). Interestingly, this peptide is recognized by the 30-2 antibody as well, since it contains Lys 90, a critical residue for 30-2 binding (Eastman, S., et al., manuscript submitted for publication, and data not shown). All CLIP-specific T cell hybrids demonstrated the same pattern of reactivity to CLIP peptide variants, but different sensitivity (data not shown).

Binding of CLIP Peptide Variants to I-A^b Molecule. Since truncated and mutated variants of CLIP may differ in their ability to bind I-A^b, we compared their ability to compete for I-A^b with titrated amounts of a known I-A^b–binding peptide, Eα52-68. Eα52-68 binding to I-A^b was measured using Eα52-68/I-A^b complex–specific mAb YAe (21). The long Ii81-104 peptide (CLIP) proved to be an efficient I-A^b binder (Fig. 4). NH_2- and COOH-terminal truncation variants Ii85-104, Ii91-104, Ii85-99, Ii89-101, and Ii81-99 bind to I-A^b with similar efficiency comparable to that of CLIP. Similarly, single amino acid substitutions Gln100 → Arg, Ala101 → Pro, Leu102 → Met, and Pro103 → Ser in Ii91-104 peptide did not affect its I-A^b–binding capacity (Fig. 4). Since Ii91-104 and Ii81-99 peptides bind to I-A^b, we suggest that the 91-99 region they share is responsible for their binding to MHC class II. Indeed, further truncations reaching into the 91-99 region from both ends sharply reduced their ability to compete for I-A^b. Peptides Ii81-93 and Ii81-91 were very inefficient competitors in this assay (Fig. 4). To directly demonstrate interaction of this region of Ii with the I-A^b molecule, we synthesized the Ii91-99 peptide and tested it in an Eα52-68 peptide competition assay. As shown in Fig. 4, this peptide competes for I-A^b. This result indicates that Ii91-99 peptide is able to

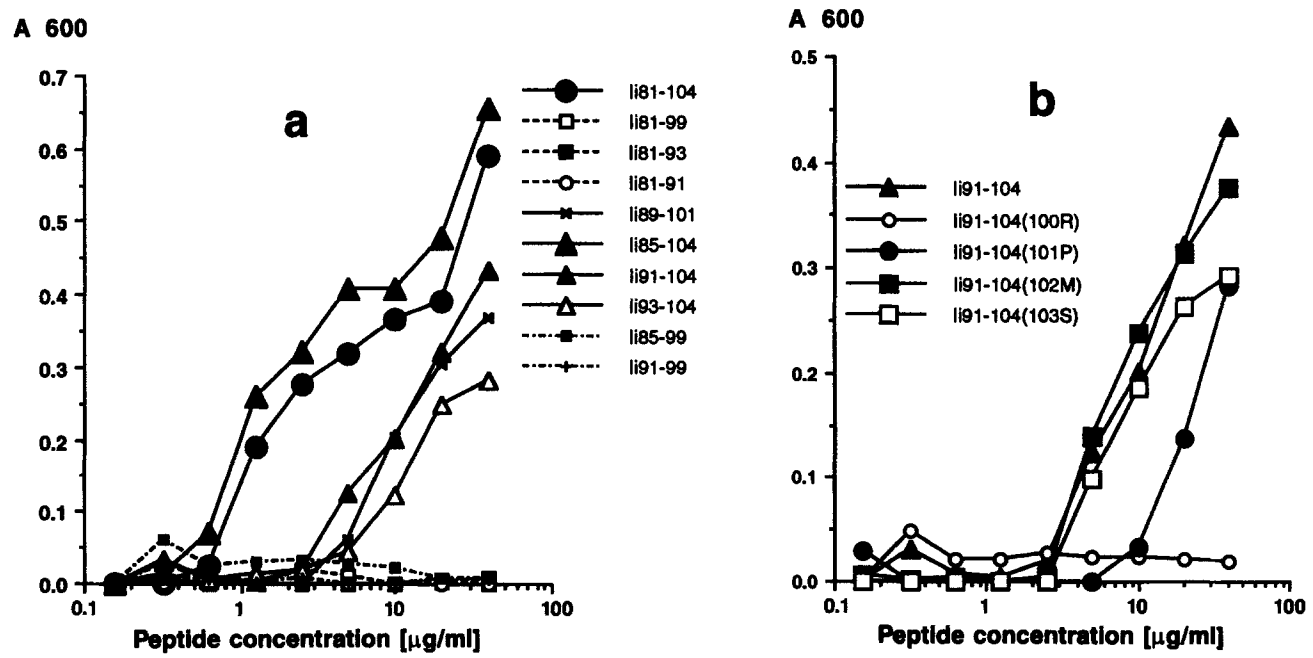


Figure 3. T cell recognition of CLIP peptide variants. (*a*) The CLIP-specific T cell hybrid 51.31 recognizes NH_2-truncated forms of synthetic CLIP peptide (*Ii89-104*, *Ii89-101*, *Ii91-104*, and *Ii93-104*), but not COOH-truncated forms of CLIP (*Ii81-99*, *Ii81-93*, *Ii81-91*, *Ii85-99*, and *Ii91-99*). (*b*) T cell responses to single amino acid substitution variants of Ii91-104 peptide. T cell response was measured as above.

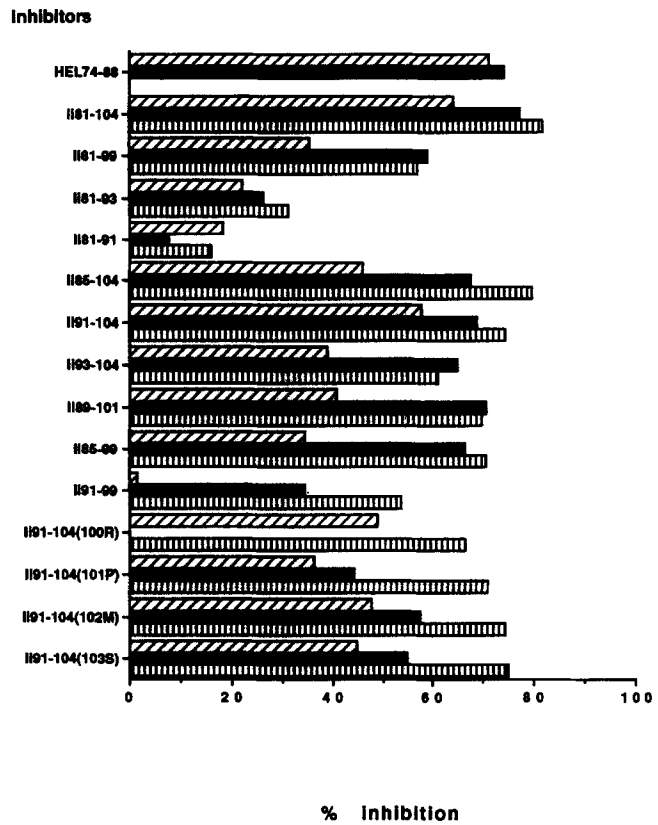


Figure 4. Binding of CLIP and its variants to I-A^b. Competition of synthetic CLIP peptide variants with Eγ2-68 peptide for I-A^b binding. Eα52-68 peptide binding to I-A^b was measured by YAe antibody staining of splenic B cells from C57BL/6 mice pulsed with 20 μM Eα52-68 peptide in the presence of 60, 200, and 600 μM inhibitor peptides. I-A^b–binding HEL74-88 peptide^{AU22} was used as a positive control for inhibition. The data are presented as percent inhibition of peptide binding calculated as (mean fluorescence intensity [MFI] in the presence of inhibitor-background): (Mean Fluorescence Intensity [MFI] in the absence of inhibitor-background) 100. Inhibition values <20% are considered not significant since this level of inhibition can be achieved at a high concentration of nonspecific moth cytochrome C peptide (MCC81-104). ▨, 3× inhibitor; ■, 10× inhibitor; ▥, 30× inhibitor.

bind to MHC class II molecule and to prevent antigenic peptide binding in the peptide-binding groove.

30-2 Antibody and T Cell Epitopes Are Expressed on the Same I-A^b Molecule. Mapping of T cell recognition site within CLIP to Gln 100 and similar mapping of the 30-2 antibody site to Lys 90 (Eastman, S., et al., manuscript submitted for publication) does not exclude the possibility that T cell and 30-2 epitopes are expressed on different rather than on the same I-A^b molecule. For example, it is possible that the T cell epitope is expressed after an I-A^b molecule re-binds free CLIP in the peptide-binding groove, whereas the 30-2 epitope may be expressed by an I-A^b molecule with the CLIP region of Ii still attached after removal of the rest of the Ii. To address this question, we assayed the ability of MHC class II molecule/CLIP complexes captured with the 30-2 mAb to stimulate CLIP-specific T cell responses. I-A^b complexes from T2-I-A^b cell lysates were captured on plates coated with 30-2 antibody or the I-A^b–specific antibodies AF6-120.1.2, M5/114, and Y3P, and were incubated with CLIP-specific T cells (Fig. 5). Class II/CLIP complexes captured on 30-2–coated plates, as well as on

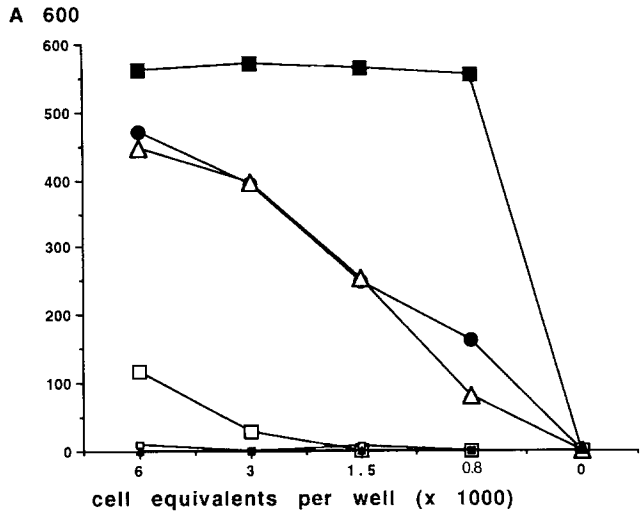


Figure 5. T cells recognize plate-captured CLIP/I-A^b complexes. 51.24 T hybrid response to total I-A^b and CLIP/I-A^b complexes from T2-I-A^b cell lysates bound to plates coated with 30-2 (*filled circles*), anti-I-A^b mAbs M5/114 (*filled squares*), Y3P (*open squares*), and AF6 (*open triangles*). As a control, anti-human Ig κ chain (*small squares*) and anti-human TfR (*small triangles*) antibodies were used. Additionally, AF6, M5/114, Y3P, and 30-2-coated plates were preincubated with T2-I-A^k lysates. The response to all dilutions of T2-I-A^k lysate was <OD 0.10 (represented by 100 on the scale). T cell response was measured as above.

AF6-120.1.2- or M5/114-coated plates elicited a strong T cell response. This suggests that 30-2-reactive I-A^b molecules are recognized by CLIP-specific T cell hybrid. Quantitative differences in T cell response to CLIP/I-A^b complexes captured by these noninhibitory antibodies most likely reflect differences in their affinities. T2 cells and T2-I-A^b transfectants express TfR and surface Ig, and they can be stained with mAbs against these molecules (data not shown). Anti-Ig κ and anti-TfR mAbs were therefore used to control for the nonspecific binding of I-A^b molecules to antibody-coated plates. As shown in Fig. 5, there was no T cell stimulation detected on plates coated with either control antibody. Similarly, I-A^k molecules from T2-I-A^k lysates captured on plates coated with AF6-120.1.2 (recognizing I-A^k as well as I-A^b) did not induce any measurable IL-2 production. Identical results were obtained with several other CLIP-specific hybrid T cell lines (data not shown). The low level of the response to complexes captured on the Y3P antibody at the highest concentration of cell lysates is probably caused by some minute aggregation of I-A^b in the lysates.

Potential aggregation of MHC class II molecules giving rise to mixed 30-2^+ and 30-2^- I-A^b aggregates could account for the observed T cell responses to 30-2-captured I-A^b molecules. To rule this out, we performed a sandwich ELISA using the same I-A^b-specific antibody as a first antibody immobilized on the solid phase and as a second antibody in the fluid phase to detect homoaggregates. This immunochemical approach did not reveal any aggregation of I-A^b molecules, since homologous combination of plate-immobilized and biotinylated 30-2, AF6-120.1.2, and another I-A^b-specific antibody, Y3P, did not detect any signal in T2-I-A^b lysates above control T2 lysates incubated with the corresponding plates (Fig. 6 *a–c*). In contrast, heterologous combinations of antibodies readily detected I-A^b and CLIP/I-A^b complexes (Fig. 6 *d–g*). In addition, we did not observe CLIP-specific T cell responses on YA*e*-coated plates incubated with I-A^b-transfected Sweig cell lysates expressing both Eα(DRα)52-68/I-A^b and CLIP/I-A^b complexes (data not shown). Therefore, we conclude that non-

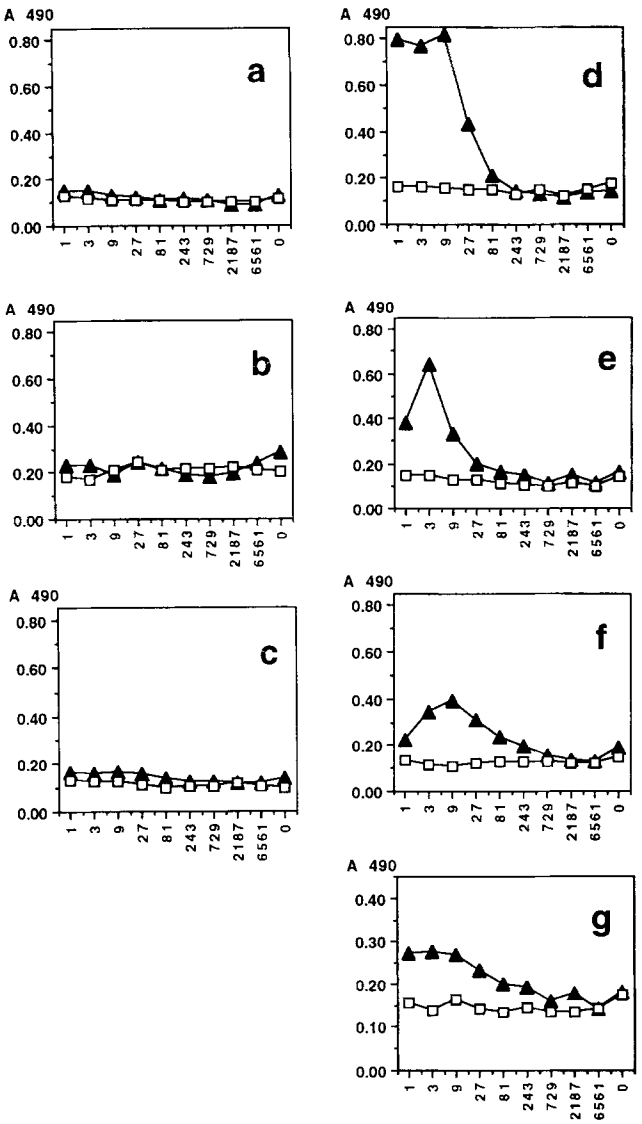
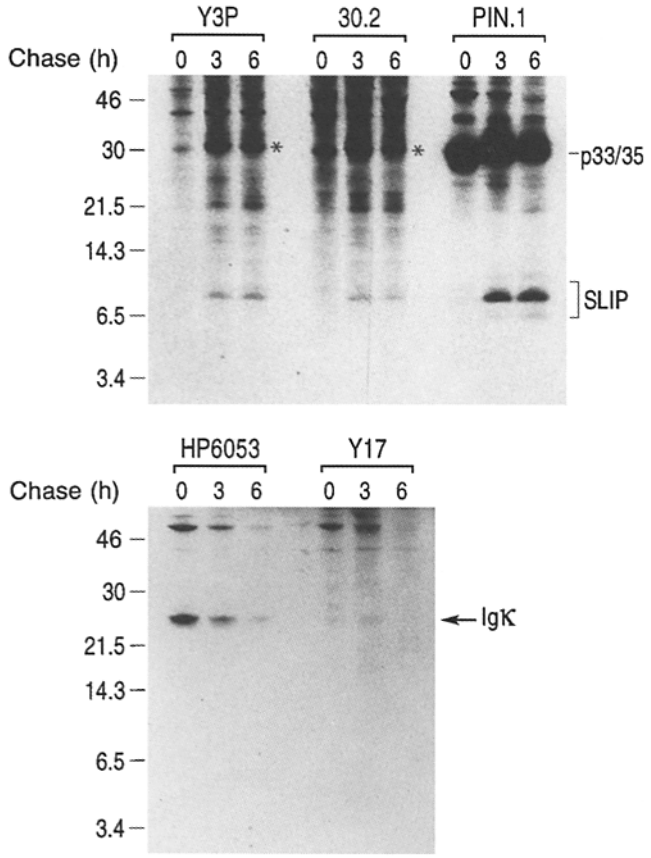


Figure 6. Analysis of homoaggregation of CLIP/I-A^b complexes and I-A^b molecules captured on antibody-coated plates by a sandwich ELISA. Determination of I-A^b from T2-I-A^b cell lysates on Y3P-coated plates using biotinylated Y3P (*a*) and AF6 (*d*) antibodies. Determination of CLIP/I-A^b complexes from T2-I-A^b cell lysates on 30-2-coated plates using biotinylated 30-2 (*b*) and AF6 (*e*) antibodies. Determination of I-A^b and CLIP/I-A^b complexes from T2-I-A^b cell lysates on AF6-coated plates using biotinylated AF6 (*c*), Y3P (*f*), and 30-2 (*g*) antibodies. Ordinate axis represents the dilution of cell lysates. *Filled triangles*, T2-I-A^b cell lysates; *squares*, T2 cell lysates (control).



overlapping 30-2 and T cell epitopes are expressed on the same population of I-A^b molecules associated with CLIP.

I-A^b Complexes with ~12-kD Ii Fragments Express 30-2 and T Cell Epitopes. Since the T and B cell epitopes of CLIP/I-A^b are independent, 30-2 antibody and T cell hybrids were used for analysis of conformation of CLIP/I-A^b and for identification of CLIP precursor(s) among larger fragments, i.e., LIP and SLIP, which are produced during endosomal degradation of Ii. Previously, we have shown that 30-2 antibody does not recognize I-A^b molecules associated with intact Ii (Eastman, S., et al., manuscript submitted for publication). To reveal expression of the 30-2 epitope by complexes of LIP and SLIP with I-A^b, we performed immunoprecipitation experiments in ^{35}S-methionine/cysteine pulse-labeled T2-I-A^b cells treated with leupeptin. SDS-PAGE analysis in Tris-tricine gels, which allow for better resolution of polypeptides with low molecular masses, revealed identical ~12-kD bands in 30-2, Y3P, and PIN.1 immunoprecipitates from drug-treated

Figure 7. CLIP/I-A^b-specific antibody 30-2 recognizes complexes of I-A^b with Ii fragments $SLIP^{AU27}$. SDS-PAGE analysis [^{35}S]methionine/cysteine pulse-labeled T2-I-A^b cells treated with leupeptin (100 μg/ml) and immunoprecipitated with 30-2, Y3P, and PIN.1 antibodies after 0, 3, and 6 h of chase. Immunoprecipitates with Y17 and anti-human Ig κ were used as controls. Samples were boiled and run over 17.5% Tris-tricine gels. Asterisk shows the position of α and β chains in the gel.

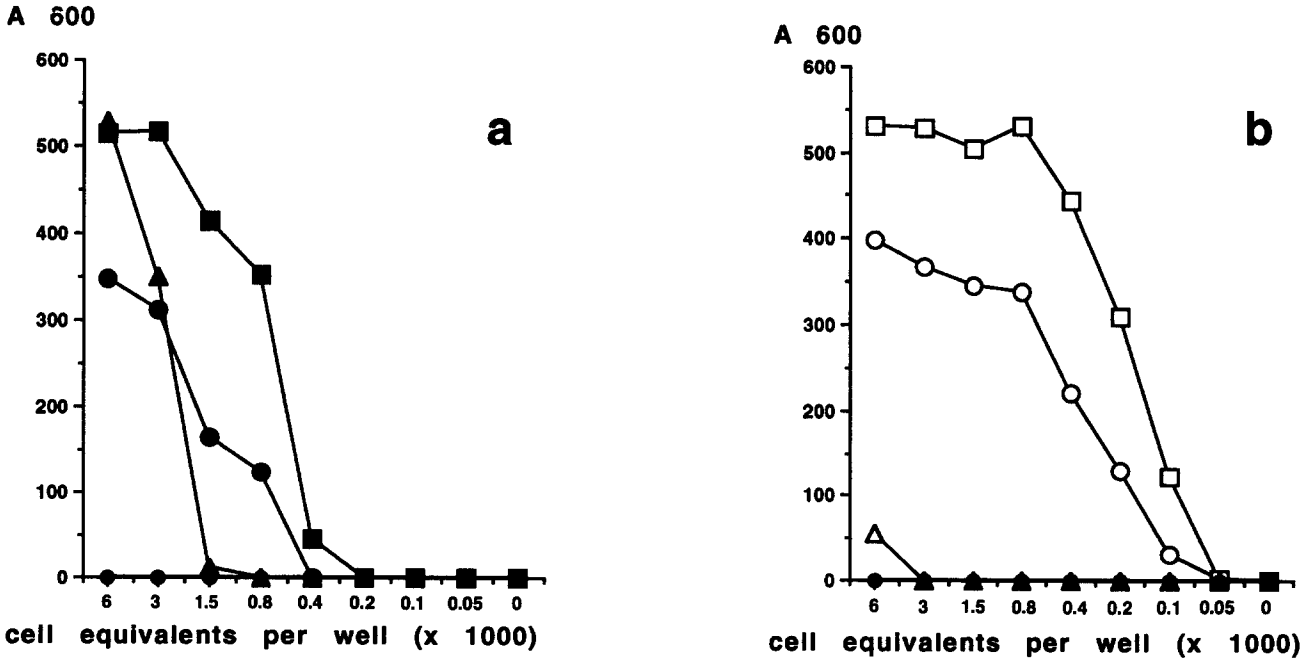


Figure 8. CLIP-specific T cells recognize complexes of I-A^b with ~12-kD fragments of Ii SLIP. 51.24 T hybrid response to SLIP/I-A^b complexes from (*a*) lysates of leupeptin-treated T2-I-A^b cells, depleted with BU45 mAb and captured on PIN.1-coated plates (*filled triangles*), 30-2–coated plates (*filled circles*), and M5/114-coated plates (*filled squares*). (*b*) T cell response to complexes from lysates of untreated T2-I-A^b cells captured on PIN.1- (*open triangles*), 30-2- (*open circles*), and M5/114- (*open squares*) coated plates. Anti–human Ig κ chain (*small circles*) and anti–human TfR (*small squares*) antibodies were used as controls. Additionally, M5/114-, PIN.1-, and 30-2–coated plates were preincubated with T2-I-A^k lysates. The response to all dilutions of T2-I-A^k lysate was ≤OD 0.10 (represented by 100 on the scale). T cell response was measured as above.

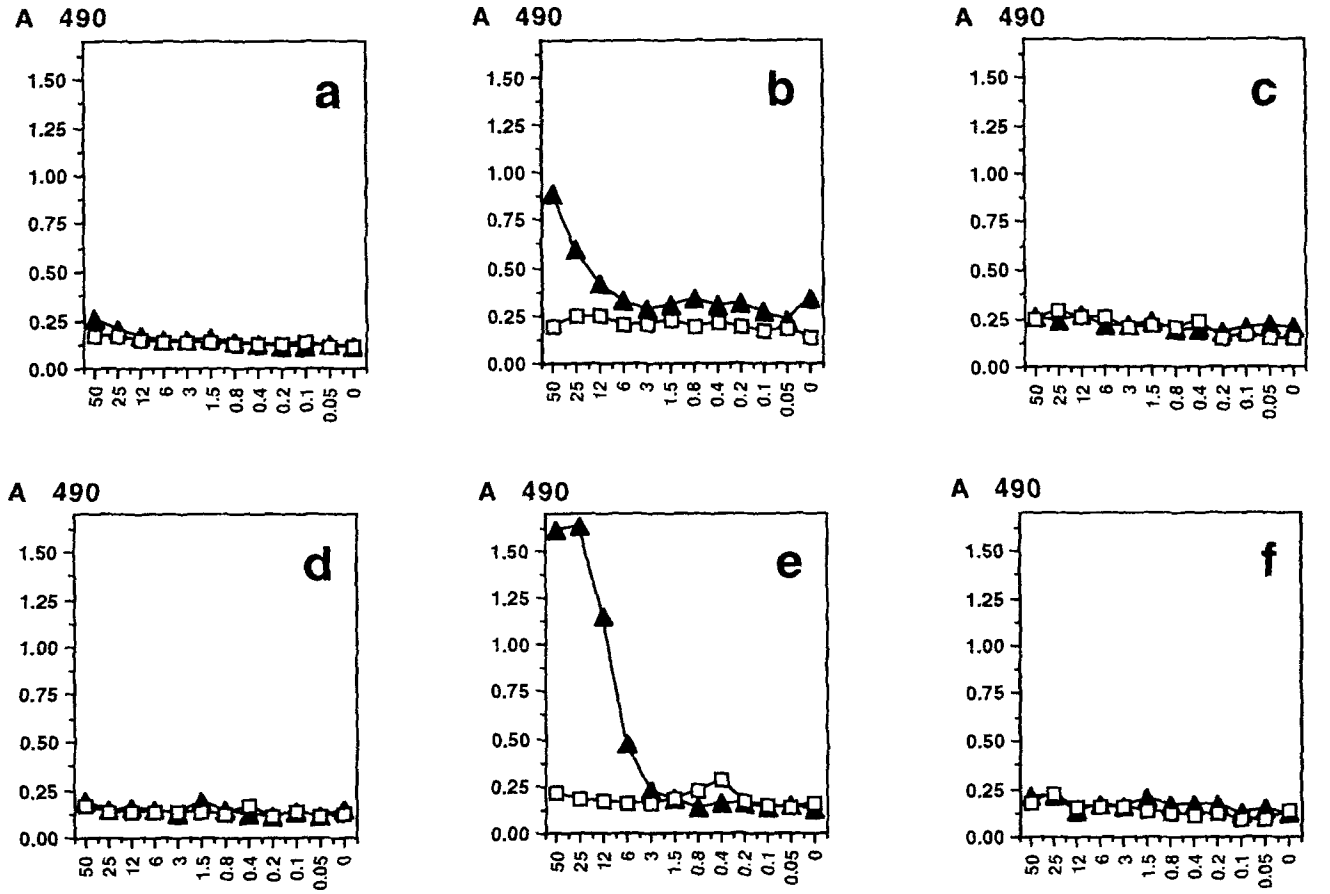


Figure 9. Analysis of homoaggregation of SLIP/I-A^b complexes and I-A^b molecules in lysates of leupeptin-treated T2-I-A^b cells by sandwich ELISA. Determination of SLIP/I-A^b complexes in leupeptin-treated T2-I-A^b cell lysates depleted with BU45 mAb on 30-2-coated plates using biotinylated 30-2 (*a*), AF6 (*b*), and Y3P (*c*) antibodies. Determination of I-A^b in the same cell lysates on Y3P-coated plates using biotinylated Y3P (*d*), AF6 (*e*) and 30-2 (*f*) antibodies. *Filled triangles*, T2-I-A^b cell lysates; *open squares*, T2-I-A^b cell lysates; *open squares*, T2-I-A^k cell lysates (control).

cells after 0, 3, and 6 h of chase (Fig. 7). Since PIN.1 antibody precipitates intact Ii as well as LIP and SLIP fragments containing an intact cytoplasmic tail, the observed 12-kD fragments in 30-2 and Y3P as well as in PIN.1 immunoprecipitates can be identified as SLIP. Anti-human Ig κ chain antibody and irrelevant antibody Y17 used as controls did not precipitate ~12-kD polypeptides. Similarly, these bands were not observed in anti-TfR precipitates (data not shown). Therefore, SLIP/I-A^b complexes express the 30-2 epitope.

To demonstrate expression of the T cell epitope on SLIP/I-A^b complexes, we tested T cell responses to these complexes captured on PIN.1-coated plates. As a source of SLIP/I-A^b complexes we used lysates of T2-IA^b cells enriched for these complexes by overnight culture in the presence of 100 μg/ml of leupeptin. To avoid potential competition of intact Ii with SLIP fragments for the binding to PIN.1-coated plates, lysates were extensively depleted of intact Ii by treatment with BU45 antibody, which recognizes the lumenal portion of Ii and does not react with SLIP fragments and protein A–Sepharose. Western blot analysis of lysates using PIN.1 antibody showed (*a*) high level of expression of Ii fragments in the presence of leupeptin and (*b*) very efficient ~98–99% depletion of intact Ii by BU45 adsorption (data not shown). SLIP/I-A^b complexes from BU45–treated lysates captured on PIN.1-coated plates induced a strong response of CLIP-specific T cells comparable to that on M5/114 or 30-2–coated plates (Fig. 8). To rule out potential oligomerization of SLIP/I-A^b or mixed SLIP/CLIP/I-A^b complexes in the lysate of leupeptin-treated cells, we performed a sandwich ELISA analogous to that shown above on Fig. 6. Similarly, we did not get a significant signal in the ELISA using homologous (30-2/30-2-biotin, Y3P/Y3P-biotin, Fig. 9, *a* and *d*) or heterologous pairs of plate-immobilized and biotinylated antibodies with overlapping epitopes (30-2/Y3P-biotin and Y3P/30-2-biotin, Fig. 9, *c* and *f*). In contrast, a strong signal was detected with heterologous pairs of antibodies with nonoverlapping epitopes (30-2/AF6-biotin and Y3P/AF6-biotin, Fig. 9, *b* and *e*). These results, as well as low response to I-A^b/Ii complexes from nontreated cells captured on PIN.1 (Fig. 8 *b*) antibody, argue against aggregation of SLIP/I-A^b complexes. It is not unexpected, since Lip/MHC class II nonamers dissociate in nonionic detergents

(23a). Thus, we conclude that both T cell and 30-2 epitopes are expressed by SLIP/I-A^b complexes, which strongly suggests that these complexes are precursors of CLIP/I-A^b complexes.

Discussion

In this study, we demonstrated for the first time that T cells recognize complexes of MHC class II molecules and Ii-derived CLIP peptides in a manner indistinguishable from that of conventional antigenic peptides. T cell hybrids recognizing human CLIP bound to I-A^b generate a strong response to normal B cell lines of human or mouse origin that coexpress human Ii and I-A^b (Fig. 1). In addition, the human presentation-deficient mutant cell line T2 and its parental cell line, T1, transfected with I-A^b present this complex very efficiently to CLIP-specific T cells. These findings are in agreement with our studies of the expression of CLIP/I-A^b complexes in mutant and wild-type cells using the mAb 30-2. This antibody specific for human CLIP/I-A^b complexes cross-reacts with corresponding synthetic and endogenous mouse peptides bound to I-A^b (Eastman, S., et al., manuscript submitted for publication). Immunohistochemical and biochemical studies in C57BL/6 mice and bone marrow chimeras using the 30-2 antibody revealed expression of these complexes on the surface of normal antigen-presenting cells in spleen, lymph nodes, and thymus (Farr, A., and A. Yu. Rudensky, manuscript in preparation). Polyclonal T cells from C57BL/6 mice immunized with the human CLIP develop a relatively low but significant in vitro proliferative response to this peptide (stimulation indexes 2–6). In contrast, T cell responses to the mouse CLIP (Ii81-104 peptide), as well as to its short variant Ii85-99 (12), were not detected (data not shown). Since mouse Ii peptides bind to I-A^b with a comparable or even higher affinity than corresponding human Ii peptides (Eastman, S., et al., manuscript submitted for publication), this indicates that T cells are tolerant to mouse Ii peptides.

The lack of T cell response to mouse Ii peptides allowed us to study the fine specificity of CLIP-specific T cell hybrids by replacing nonconserved amino acid residues in human CLIP sequence with corresponding mouse residues. This analysis demonstrated that all T cell hybrids recognize the same residue of the CLIP peptide that is distinct from that recognized by the 30-2 antibody. Previously, we have identified the a lysine residue at position 90 of the human Ii as a critical residue for 30-2 antibody recognition (Eastman, S., et al., manuscript submitted for publication). In contrast, T cell specificity is confined to the COOH-terminal portion of CLIP, particularly to glutamine at position 100. Interestingly, antibody and T cell epitopes are not overlapping, since the 30-2 antibody does not block T cell response to CLIP presented by I-A^b–positive APCs. In addition, CLIP/I-A^b complexes bound to 30-2–coated plates are efficiently recognized by T cells, which further proves that both T cell and antibody epitopes are expressed on the same MHC class II molecule. Importantly, T cell and antibody recognition are both critically dependent, not only on the peptide, but also on the MHC class II allele. Complexes of CLIP with I-A^d and I-A^{bm12} are not recognized by the 30-2 antibody or the T cells (Eastman, S., et al., manuscript submitted for publication, and Fig. 1). The latter differs from I-A^b at three amino acid residues at positions 67, 70, and 71 (24). How can we explain the lack of a steric inhibitory effect of the 30-2 antibody on CLIP-specific T cell response, as well as the effect of bm12 mutation on both TCR and antibody recognition? First, the 30-2 antibody may bind from the side of the MHC class II–binding groove with both CLIP residues Lys 90 and some I-A^b residues contributing to the binding site, whereas the TCR may interact with the I-A^b/CLIP complex from the top of the groove. It is possible that distinct I-A^b residues affected by bm12 mutation contribute differently to T cell and antibody epitopes. Another possible interpretation for these findings, which does not exclude the first one, is an indirect effect of certain amino acid residues in the CLIP (e.g., Lys 90) or in the I-A^b (bm12 substitutions) on the conformation of the entire complex. Structural analysis is required to give a final answer to this question.

Our analysis of the CLIP binding to I-A^b using the original peptide and its truncation variants demonstrated that the nonamer corresponding to the 91-99 region of Ii can bind to I-A^b and compete for the presentation with the antigenic Eα52-68 peptide. This part of the CLIP sequence is identical in the mouse, human, and rat Ii (25–28), and it suggests that the conserved 91–99 region of the Ii is the MHC class II–binding site. These results are in agreement with several studies of Ii/MHC class II association using a series of COOH-terminal deletion variants of Ii coexpressed with human and mouse MHC class II (17–19). Freisenwinkel and coauthors demonstrated that the 80–110 region of the Ii is necessary for the Ii association with MHC class II (17). These authors postulated binding of CLIP at a position distinct from the peptide-binding groove. In contrast, Romagnoli and Germain (18) suggested a model of CLIP binding inside the groove while CLIP is still a part of the Ii1-107 fragment. The latter is supported by our findings. A recent study by Bijlmakers and colleagues further narrowed the site of Ii/MHC class II interaction to residues 96–104 (19). In agreement with these data, we did not find significant binding of the Ii81-93 peptide, in contrast to 93-104 peptide, which was able to bind to I-A^b and to elicit a T cell response. These data suggest that the 91-99 peptide of the Ii may represent an MHC class II–binding site. Interestingly, the Ii89-101 peptide, which contains the MHC class II–binding 91-99 site flanked with Lys 90, Gln 100, and Ala 101, which are critical for 30-2 and T cell recognition, respectively, binds to I-A^b and is recognized by the antibody and T cell (Figs. 3 *a* and 4). Recent study by Malcherek et al. (29) provided evidence that CLIP may act as a universal groove-binding peptide, taking advantage of sequence supermotifs and allowing CLIP to bind to various MHC alleles. All previous studies, as well as this one, do not exclude contribution of other regions of the Ii, such as the transmembrane region or the more COOH-terminal parts of the lumenal portion

of Ii, to its binding to MHC class II molecule. These additional sites may be critical for Ii binding to MHC class II alleles with a relatively low affinity for CLIP, e.g., HLA-DR4 or DR11 (15, 20).

Our results suggest that the COOH-terminal portion of the CLIP region, which includes T cell contact residue Glu 100, binds in the groove of the I-A^b molecule. It seems likely that the orientation of NH_2 and COOH termini of CLIP associated with MHC class II follows that of antigenic MHC-binding peptides. In this case, the very NH_2-terminal portion of CLIP more distant from the MHC-binding site is likely to be exposed and recognized in an MHC-independent manner, whereas Lys 90 is still in the groove or in its immediate proximity such that it is recognized by the 30-2 antibody in an MHC-restricted fashion. This model is supported by the observation that the Cer-CLIP.1 antibody, recognizing free as well as MHC-bound CLIP independently of an MHC allele (30), does not react with any of NH_2-terminal truncation variants of CLIP used in this study (Cresswell, P., personal communication). In addition, the binding of CLIP to I-A^b molecules protects CLIP containing both T cell and antibody recognition sites from cleavage by trypsin, similarly to a single amino acid substitution variant of antigenic Eα52-68 peptide (57 Glu→Lys) containing a single trypsin cleavage site in the middle of the sequence (Goldrath, A. W., and A. Yu. Rudensky, unpublished observation). Similarly, CLIP peptide bound to the HLA-DR3 molecule was protected from proteolytic degradation by a mixture of cathepsin B and D (20).

The T cell recognition of CLIP/MHC class II complexes raises a question concerning the mechanism responsible for generating of these complexes. In other words, is CLIP a remnant of the Ii containing the MHC class II–binding site that remains associated with MHC class II after the rest of the Ii is cleaved off, or is this fragment first released by proteases as a free peptide which then binds in the groove of MHC class II molecule? Generation of CLIP/I-A^b–specific T cells and mAb 30-2 provided us with a tool to compare the conformation of I-A^b molecule associated with free CLIP peptide and I-A^b complexes with larger fragments of Ii containing the CLIP region. These fragments, 21-kD LIP and 11–14-kD SLIP, were first described in B cell lines treated with the cysteine proteinase inhibitor leupeptin (10, 11). LIP and SLIP fragments stay associated with MHC class II molecules in the endosomal compartment, thus preventing class II export to the cell surface (31). Both 30-2 antibody and CLIP-specific T cells recognize complexes of I-A^b with ~12 kD SLIP fragments (Figs. 7 and 8). Thus, SLIP fragments contain CLIP region bound to MHC class II molecule in a conformation indistinguishable from that of free CLIP peptide. This indicates that (*a*) CLIP is a remnant of Ii and (*b*) SLIP/I-A^b complexes are precursors of CLIP/I-A^b complexes. SLIP fragments are generated in late endosomes and in a dense endosomal compartment, MIIC (reference 11 and J. Blum and Rudensky, A., manuscript in preparation). Recent studies suggested that the latter compartment is a critical intracellular site for MHC class II complex assembly with peptides derived from both endogenous and exogenous antigens (32–35). Therefore, it is likely that the bulk of CLIP/MHC class II complexes is generated in the same compartment. Further experiments with CLIP-specific T cell hybrids and the 30-2 antibody using subcellular fractionation will directly test this prediction.

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