Class II-Associated Invariant Chain Peptide-Independent Binding of Invariant Chain to Class II MHC molecules

Wesley P. Thayer, Leszek Ignatowicz, Dominique A. Weber and Peter E. Jensen

*J Immunol* 1999; 162:1502-1509; ;
http://www.jimmunol.org/content/162/3/1502

---

**References**
This article cites 62 articles, 32 of which you can access for free at:
http://www.jimmunol.org/content/162/3/1502.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Class II-Associated Invariant Chain Peptide-Independent Binding of Invariant Chain to Class II MHC molecules

Wesley P. Thayer,* Leszek Ignatowicz,† Dominique A. Weber,* and Peter E. Jensen2*

The class II-associated invariant chain peptide (CLIP) region of invariant chain (II), has multiple important functions in the MHC class II Ag-processing pathway (1–3). II homotrimers rapidly assemble with newly synthesized class II αβ heterodimers in the endoplasmic reticulum (ER) (4, 5) to form nonameric complexes (6) that are transported through the Golgi and targeted to endosomal compartments. II is released after sequential cleavage by endopeptidases (7–10), leaving only a fragment encoded by exon 3, class II-associated invariant chain peptides (CLIP), which is protected from proteases because it is largely buried in the peptide-binding groove (11). This last fragment is removed by HLA-DM, which catalyzes peptide-exchange reactions in class II molecules (12–14). II facilitates the initial assembly and transport of DM, which catalyzes peptide-exchange reactions in class II mol-

Copyright © 1999 by The American Association of Immunologists 0022-1767/99/$02.00

The present study was initiated to investigate the role of the CLIP sequence and its affinity for the peptide-binding site in mediating assembly and transport of αβII complexes. The results indicate that a mutant II protein containing a CLIP sequence designed to have high affinity for the peptide-binding site can be stable in SDS (31), a property conferred by occupancy of the binding site (32). This would explain the inability of αβII to bind peptide Ags. Class II molecules with unoccupied binding sites are less stable and more likely to misfold and denature (33–36). Thus, binding-site occupancy may be the mechanism through which II promotes the folding and early transport of some class II heterodimers. Indeed, Zhong et al. (37) demonstrated that groove occupancy alone is sufficient for promoting transport of class II molecules through the secretory pathway. Given the apparent importance of binding-site occupancy, it is remarkable that the conserved sequence of CLIP can appropriately interact with all class II molecules despite extensive polymorphism in the binding site. Class II molecules have been shown to differ widely in their affinities for CLIP (38, 39).

The present study was initiated to investigate the role of the CLIP sequence and its affinity for the peptide-binding site in mediating assembly and transport of αβII complexes. The results indicate that a mutant II protein containing a CLIP sequence designed to have very low affinity for DR1 efficiently assembles with DR1 and mediates its transport to endosomal compartments. The loss of affinity for the peptide-binding site is complemented by other interactions involving a segment of II on the C-terminal side of CLIP. We demonstrate that a C-terminal fragment of II binds stably to a site in class II molecules outside the peptide-binding groove, with no requirement for the N-terminal or CLIP regions of II. These results support the conclusion that no minimal affinity for the peptide-binding site is required for the assembly and transport of class II-II complexes, consistent with the ability of II to regulate the transport of all class II molecules regardless of affinity for CLIP.

Materials and Methods

DNA constructs

All mammalian expression constructs were cloned into pCDNA3.1 Zeo (Qiagen, Valencia, CA), and genes were sequenced. The DRα- and
β-chains, generously provided by Dr. Eric Long (National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Bethesda, MD) in vector CDMA, were subcloned into pCDNA3.1 Zeo by using 5‘ blunt and 3‘ Xhol digested PCR product generated with the following primers: forward: α-F, 5‘-GAAGAATGGCTCAGGTGATCTCCTG-3‘ and β-F, 5‘-CAGCATTGGTTGTCAGCTCCTG-3‘; reverse: CDMA-R, 5‘-CACGCGATCCCAAGCATTTTTGAGGG-3‘ and the internal reverse primer was 5‘-GACCTGCTGACCTTCTGAGAGG. A complete list of the plasmids was the kind gift of Dr. Ronald Germain (NIAID, Bethesda, MD) and was cloned into pCDNA3.1 Zeo using two primers (forward - GGCAACGTTTCTACGCTTCCTTCTCTAAAGC; and reverse - ATAGAAATGGCCGCGCTGAGATGC TAGTTGCTTGTTGCTC-3‘) followed by digestion with KpnI and Xhol. APβ-Eco (40) in pZol was subcloned using existing EcoRI sites.

The human lip335 construct was also provided by Dr. Eric Long. Using overlapping PCR, the murine li (mli) cytoplasmic domain (mlip335-pcEXV-3 construct provided by Dr. Jim Miller, University of Chicago, Chicago, IL) was engineered onto the human version with the following primers: pcEXV-3-F, 5‘-GGAGTTGCTTCTGCTGCCTAAGGAAG with li-mctry-RT -GGCCATCAGGCGCATCTGTTAGCTG-3‘, and the internal reverse primer was 5‘-GACCTGCTGACCTTCTGAGAGG. A complete list of the plasmids was the kind gift of Dr. Ronald Germain (NIAID, Bethesda, MD) and was cloned into pCDNA3.1 Zeo using two primers (forward - GGCAACGTTTCTACGCTTCCTTCTCTAAAGC; and reverse - ATAGAAATGGCCGCGCTGAGATGC TAGTTGCTTGTTGCTC-3‘) followed by digestion with KpnI and Xhol. APβ-Eco (40) in pZol was subcloned using existing EcoRI sites.

**Immunofluorescence**

Transfected COS were harvested by trypsinization and centrifuged onto slides using a Shandon Southern Cytospin, fixed in 95% ethanol at −20°C, and stained with L243 (DR) followed by goat anti-mouse FITC. Cells were visualized on a Leica microscope and captured using a MCID Image Analysis System (Image Research, Ontario, Canada) at ×40.

**Purification of li ectodomains**

Escherichia coli strain, XL1-Blu (Strategene, La Jolla, CA), was transformed with each pQE-9 construct. Bacteria were incubated at 37°C in LB broth containing 50 µg/ml of ampicillin, and protein expression was induced with 1 µM isopropyl β-D-thiogalactoside at OD600 of 0.4. Four hours after induction, cells were harvested, and the bacterial pellet was resuspended in PBS with protease inhibitors. The cells were sonicated, and the lysate was cleared for 10 min at 10,000 × g. Cleared lysate from 800 ml of induced culture was mixed with 2 ml of Ni-nitrilotriacetic acid (NTA) resin (Qiagen). The resin was washed four times with PBS containing 10 mM imidazole and eluted in PBS and 250 mM imidazole. Ion-exchange chromatography of histidine-tagged fragments was performed using a Bio-Scale Q2 anion exchange column (Bio-Rad, Hercules, CA) and Bio-Rad Biologic high resolution liquid chromatography system. A linear gradient from 0.02 to 1.0 M NaCl and 50 mM Tris (pH 7.0) was used, and the major peak was collected for further study. Purified proteins were labeled with either fluorescein isothiocyanate or biotinamidocaproyl N-hydroxysuccinimide ester. Briefly, either reagent, dissolved in DMSO at 2 mg/ml, was added to the PBS-dialyzed protein at a 2:1 molar ratio, rocking 1 h at room temperature. The reaction was terminated by the addition of Tris (pH 8.0) for 10 min followed by dialysis.

**Abs, DR, DM, and peptides**

The mAbs La243 (43), Tu36 (44), Yae (45), and IN1 (46) were all purified from hybridoma supernatant using protein A- or G-Sepharose affinity chromatography. Bu45 was obtained from The Binding Site (Birmingham, U.K.). HLA-DR1 and HLA-DM were purified as previously described (47). Peptides were synthesized by fluorenyl methoxycarbonyl chemistry with a Ranin Instruments (Emoryville, CA) Synthepator peptide synthesizer, and certain ones were labeled with a biotin at the major peak was collected for further study. Purified proteins were labeled with either fluorescein isothiocyanate or biotinamidocaproyl N-hydroxysuccinimide ester. Briefly, either reagent, dissolved in DMSO at 2 mg/ml, was added to the PBS-dialyzed protein at a 2:1 molar ratio, rocking 1 h at room temperature. The reaction was terminated by the addition of Tris (pH 8.0) for 10 min followed by dialysis.

**Binding assays**

Peptide affinity for DR1 was measured by competition inhibition assays in which 50 nM DR1 was incubated with 0.5 µM biotin-CLIP(81–104) or various concentrations of biotin-hIi(103–216) in 0.2% Nonidet P-40, 100 mM citrate/phosphate (pH 5.0) for 18 h at 37°C with varying concentrations of competitor peptide. After incubation, the samples were captured on microtiter assay plates coated with L243, and biotinylated ligand binding to DR1 was quantified with a Tosohaas Binding System (Imaging Research, Ontario, Canada) at 37°C.

### Transient expression

COS 7 cells were transiently transfected with cDNA constructs using the DEAE-dextran method as described (41). Briefly, 5 × 10⁶ cells were plated per 60-mm tissue culture dish with DMEM and 10% FCS 24 h before transfection. Cells were washed twice with DMEM and 10 mM HEPES, and each dish was incubated in 2 ml of DMEM and 10 mM HEPES containing 500 µg of DEAE-dextran, 100 µM chloroquine, and DNA (0.5 µg of each class II α- and β-chain construct + 1 µg of various li constructs). After 3 h at 37°C, the cells were treated with 10% DMSO in DMEM and 10 mM HEPES for 1–2 min at room temperature, then incubated 4 h in DMEM and 10% FCS before use in immunoprecipitation or immunofluorescence experiments.

### Metabolic radiolabeling, immunoprecipitation, and gel electrophoresis

Transfected COS plates were each washed twice, Cys/Met depleted for 30 min at 37°C with 3 ml of Met/Cys-deficient DMEM-5% dialyzed FCS, then labeled for 30 min in 2 ml Met/Cys-deficient DMEM-5% dialyzed FCS medium containing 0.05 mCi/ml of [35S]-trans Cys/Met (Trans-35S-Label, ICN Pharmaceuticals, Irvine, CA). Plates were subsequently washed, harvested by trypsinization, and lysed on ice for 1 h in 0.5% Nonidet P-40 lysis buffer, 0.15 M NaCl, 50 mM Tris (pH 7.5), 0.01% Azide, and a protease inhibitor mixture (42). The precleared detergent lysates were split and immunoprecipitated with mAb prebound to protein A- or G-Sepharose beads (Sigma, St. Louis, MO) for 1 h, rocking at 4°C. Beads were preloaded by incubating 1.0 ml of supernatant or 10 µg of purified mAb with 40-µl beads for 1 h, rocking at 4°C, then washed three times in PBS. After incubating with lysates, beads were washed four times with 500 µl of 0.2% Nonidet P-40 wash buffer, 0.15 M NaCl, 50 mM Tris (pH 7.0), and 0.5 mM EDTA. Samples were eluted with 2% SDS and 5% 2-ME buffer by boiling 5 min and resolved using 12% polyacrylamide gels.

To investigate the role of CLIP affinity for the class II peptide-binding site in li function, variants were generated in which the
sequence of CLIP was mutated to decrease or increase its affinity for DR1. The low-affinity sequence (BAD-CLIP) has five substitutions at anchor positions (11) designed to substantially reduce affinity for DR1, which strongly prefers peptides with a large hydrophobic residue in the dominant pocket, P1 (50) (Fig. 1A). Positively charged Arg at P4 should be disfavored because of charge repulsion from Argb71, and Thr is not optimally accommodated in the shallow P6 pocket (50). Similarly, Glu is inappropriate for the environment provided by the hydrophobic P9 pocket. A synthetic BAD-CLIP peptide was observed to have a substantially reduced affinity for DR1 as determined in competition-inhibition binding assays (Fig. 2A). The high affinity mutant CLIP sequence (M91Y) was previously shown to increase the stability of the DR1-peptide complex by 160-fold, despite having little effect on the apparent affinity measured in competition assays (47) (Fig. 2A).

Full-length mutant human p31Ii cDNA constructs, encoding the mouse Ii cytoplasmic domain as a marker, were generated by site-directed mutagenesis and cloned into a mammalian cell expression vector (Fig. 1B). Expression was confirmed in metabolically labeled COS cell transfectants by immunoprecipitation with mAb specific for the cytoplasmic tail of mIi, IN-1 (Fig. 3A, lower panel).

DR association and function of mutant forms of Ii

Ii mutants were coexpressed in COS cells with human class II DR1α and β-chains, and proteins from pulse-labeled cells were precipitated using mAbs specific for either DR1 (Tu36) or IN-1 (Ii). Ii and Ii.M91Y associate efficiently with DR1, as expected (Fig. 3A). Surprisingly, Ii.BAD-CLIP was also observed to efficiently coprecipitate with DR1. The capacity of the mutant Ii proteins to facilitate the transport and targeting of DR to endosomal compartments was analyzed by immunofluorescence. In the absence of Ii, DR fluorescence is distributed in a fine reticular pattern (Fig. 4). DR is redistributed to endosomal compartments characterized by a discrete vesicular staining pattern after cotransfection with Ii. Both the high- and low-affinity mutant Ii proteins were also able to target DR to the endosomal pathway (Fig. 4). These results suggest that the affinity of the CLIP region has little, if any, impact on the capacity of Ii to associate with DR and direct its transport to Ag-processing compartments.

Our findings raised the concern that the BAD-CLIP sequence may have sufficient affinity to mediate stable association with the DR1 peptide-binding site despite the inclusion of inappropriate anchors and data showing low affinity in peptide binding experiments with purified DR1. To address this concern, several C-terminal deletions were made before and after the CLIP region in wt and mutant Ii (Fig. 1C). It has been shown previously that C-terminal Ii deletion mutants up to, but not including, the CLIP region associate with class II heterodimers, suggesting that CLIP is essential for stable interaction (51, 52). We confirmed this observation by demonstrating that Ii(1–103) but not Ii(1–83) efficiently coprecipitate with DR1 (Fig. 3B). However, BAD(1–103) does not associate with DR1, demonstrating that the low-affinity BAD-CLIP sequence is insufficient to mediate assembly with DR in the absence of the Ii C-terminal region. These results imply that some
portion of the C-terminal domain of Ii is able to complement the loss of affinity for the peptide-binding groove in full-length Ii.BAD-CLIP.

The Ii(103–117) region of Ii is required for CLIP-independent association with DR1

To identify the C-terminal domain of Ii responsible for association of BAD-Ii with DR1, a series of C-terminal deletions were generated (Fig. 1C) and cotransfected with DR1αβ into COS cells. By immunoprecipitation, we observed that each BAD-Ii C-terminal deletion mutant was able to associate with DR1 except for the shortest, BAD-Ii(1–103), implying that the 104–118 region is sufficient to complement the loss of affinity for the peptide-binding site (Fig. 5A). To further characterize the role of the 104–118 region, a series of wt Ii N-terminal deletions were generated that included signal peptides to direct translocation into the ER (Fig. 1C). Not only did the entire lumenal domain fragment (58–216), which contains the wt CLIP sequence, associate with DR1, but a shorter soluble fragment (103–216), which completely lacks a CLIP region, also associates with DR1 (Fig. 5B). This result clearly demonstrates that interactions involving the C-terminal domain of Ii can independently mediate stable association with DR. Further deletion of the 15-amino acid region (103–117) abrogates association, confirming that the region immediately C-terminal to CLIP is required for CLIP-independent binding (Fig. 5B).

Because fragment 103–117 is very close to the CLIP sequence, we considered the possibility that the physical orientation of Ii relative to DR may shift such that fragment 103–117 actually binds in the peptide-binding groove to mediate association with DR1. However, a synthetic Ii(103–118) peptide was observed to have very low affinity in competitive binding experiments with purified DR1 (Fig. 2B). To further address this possibility, we evaluated the ability of Ii(103–216) to associate with a class II molecule in which the peptide-binding site was occupied by a covalently tethered peptide. Ii(103–216) was observed to coprecipitate with IAb-Eα complexes immunoprecipitated with the YAe mAb, which only recognizes IAb molecules containing the Eα peptide in the peptide-binding groove (Fig. 6). The quantity of coprecipitated Ii fragment was relatively low but clearly detectable. It is possible

FIGURE 3. Effect of CLIP mutations on association of Ii with DR1. COS cells were transfected with DRαβ and the indicated full-length (A) or truncated (B) Ii constructs. Cells were labeled for 30 min with 35S-tran Cys/Met, and precleared detergent lysates were split and immunoprecipitated with mAb Tu36 (DR) or In-1 (Ii). The positions of the Ii bands are indicated by arrows. The mutant protein, Ii.BAD, was observed to have slightly slower mobility than wt Ii or Ii.M91Y on SDS-PAGE.

FIGURE 4. Ii mutants target DR1 to endosomal compartments. Transfected COS cells expressing DRαβ alone or in combination with wt or mutant Ii were harvested and centrifuged onto slides using a Shandon (Pittsburgh, PA) Southern Cytospin, fixed in 95% ethanol at −20°C, and stained with L243 (DR) followed by goat anti-mouse FITC. Cells were visualized on a Leica (Deerfield, IL) microscope, and images were captured using a MCID Image Analysis System (Imaging Research) at ×40.
that the covalent peptide linker partially interferes with Ii association or that mAb YAE has greater affinity for free Iaβ than Iaβ-Ii complexes. Nevertheless, we conclude that the 103–117 region of Ii interacts with class II molecules through a site outside of the peptide-binding groove.

**Generation and analysis of recombinant soluble C-terminal Ii fragments**

To further investigate the binding of the Ii C-terminal domain to class II molecules, soluble His-tagged 103–216 and 118–216 fragments of Ii were expressed in E. coli, isolated by Ni-chelation, and further purified by ion exchange chromatography (Fig. 7). Chemical crosslinking showed that the fragments can trimerize (Fig. 7C and data not shown), as was previously demonstrated by Park et al. (53) for rII(118–216).

Fluorescein (Fl)-labeled rII(103–216) protein was observed to bind to purified DR1 by using HPSEC to separate bound from free protein (Fig. 8A). Binding was inhibited by excess unlabeled rII(103–216) but not rII(118–216), confirming that the 103–117 region is required. No inhibition was observed with a 100-fold excess of synthetic II(103–118). Thus, this sequence is essential but not sufficient for binding. The conclusion that association occurs through interactions outside of the peptide-binding groove was confirmed by the observation that the high affinity, synthetic II(89–100) M91Y peptide did not inhibit binding. The association kinetics were reasonably rapid, with apparent saturation within 5 h (Fig. 8B). The binding kinetics, not yet analyzed in detail, may be complicated because of the capacity of rII(103–216) to form homotrimers. Saturable binding of biotin-labeled rII(103–216) could also be measured using a europium fluorescence immunoassay, with an apparent equilibrium dissociation constant in the submicromolar range (Fig. 8D). It is interesting that the binding of the Ii C-terminal domain to DR1 is highly dependent on pH, with optimal binding at neutral pH and considerably reduced binding at acidic pH (Fig. 8C). Thus, the acidic environment present in endosomal compartments may directly contribute to the release of Ii by disrupting CLIP-independent interactions involving the C-terminal domain of Ii.

**Discussion**

In this study, we observed that a mutant Ii protein, designed to have little or no affinity for the peptide-binding site of DR1, efficiently associates with DR1 and directs its transport to endosomal compartments in COS cell transfectants. The loss of affinity for the peptide-binding site was confirmed by the observation that a C-terminally truncated form of the Ii protein, BAD(1–103), which includes a mutant CLIP sequence, does not associate with DR1 in contrast to wt II(1–103). Thus, the wt but not the mutant CLIP
interactions involving other sites in Ii also participate in stabilizing affinity between CLIP and the peptide-binding site. It is likely that involving the Ii(103–118) region can compensate for a loss of ER (51, 52, 55). It is clear from the present study that interactions role in mediating the assembly of Ii with class II molecules in the cated Ii have demonstrated that the CLIP region can play a critical binding groove.

We considered the possibility that the Ii(103–118) region may directly interact with the peptide-binding site, replacing the function of the C-terminal domain of Ii. Cotransfection with C-terminally truncated forms of Ii.BAD-CLIP demonstrated that the Ii(104–117) region immediately adjacent to CLIP was required to complement loss of affinity for the peptide-binding site. Remarkably, a C-terminal fragment completely lacking the CLIP region, Ii(103–216), was observed to efficiently associate with DR1 in transfected cells. Furthermore, rli(103–216) bound specifically to purified DR1. Ii(118–216) did not associate with DR1 in cotransfection studies or experiments with purified proteins, confirming a critical role of the (104–118) region.

We considered the possibility that the Ii(103–118) region may directly interact with the peptide-binding site, replacing the function of the CLIP sequence. Several observations excluded this possibility. A synthetic peptide representing this sequence did not bind to purified DR1 in competitive binding experiments, indicating that it has very low affinity for the peptide-binding site. Immuno-precipitation experiments with the YAe mAb, which only recognizes I4 containing the Eaa peptide in the peptide-binding groove (54), demonstrated that Ii(103–216) can associate with class II molecules in which the groove is occupied by a defined peptide. Finally, a 100-fold molar excess of a high-affinity DR1-binding peptide had no effect on the binding of rli(103–216) to purified DR1. Thus, the C-terminal domain and the Ii(103–118) region must interact with a site in class II molecules outside the peptide-binding groove.

Previous studies with deletion mutants and C-terminally truncated Ii have demonstrated that the CLIP region can play a critical role in mediating the assembly of Ii with class II molecules in the ER (51, 52, 55). It is clear from the present study that interactions involving the Ii(103–118) region can compensate for a loss of affinity between CLIP and the peptide-binding site. It is likely that interactions involving other sites in Ii also participate in stabilizing αβIi complexes. The observation that Ii(103–216), but not the synthetic Ii(103–118) peptide, binds DR1 suggests that other sites in the C terminus cooperate with 103–118. This is not likely to be a result of a conformational constraint placed on 103–118 in the context of the complete C-terminal domain because the 103–118 region is also required for binding of the 1–118 N-terminal fragment of Ii.BAD-CLIP, which lacks the remainder of the C terminus. Park et al. (53) have provided evidence for another interaction site in the C terminus by demonstrating that rli(118–208) can enhance the binding of radiolabeled peptide Ag to DR1, implying a specific interaction. Our results indicate that this interaction is relatively weak compared with the binding of the larger Ii(103–216) C-terminal fragment. The results are in agreement with those of Newcomb et al. (56), who reported that a C-terminal fragment of Ii, beginning at Gly110, remains associated with DR after in vitro digestion of purified αβIi with protease K. The 110–118 segment contains a predicted N-linked glycosylation site at position 114. However, the glycosylation mutant, Ii.BAD-CLIP(N114A), was observed to coprecipitate efficiently with DR1 (data not shown), indicating that carbohydrate at this position is not required for binding.

There is also strong evidence that an N-terminal segment adja-cent to the core CLIP sequence contributes to the interaction of Ii with class II αβ (31, 57–59). Kropshofer and colleagues (57, 58) reported that N-terminal segment of CLIP, 81–89, which does not interact with pockets in the class II binding site (11), can facilitate the dissociation of CLIP from DR molecules. stumpfner and Benroch (31) provided evidence that the Ii(81–90) region can influence the conformation of the peptide-binding site and enhance the DR association of various mutant Ii constructs with deletions or substitutions in the CLIP region. Most recently, Siebenkotten et al. (59) demonstrated coprecipitation of the Ii deletion mutant Δ88–127 but not Δ81–127 with DR in COS cell transfectants. This suggests that the 81–87 region may be able to complement the loss of interactions mediated by both the core sequence of CLIP, 91–99, and the 103–118 segment identified in the present study. However, it is clear from previous work (51, 52) and the present study that interactions involving Ii(81–87) are not sufficient to...
mediate stable association with class II αβ in the absence of other C-terminal regions of Ii. The C-terminal domain appears to make a quantitatively greater contribution given that Ii(103–216) binds to DR1 with high affinity to form stable complexes, whereas binding of Ii(1–103)BAD-CLIP cannot be detected.

It is likely that non-CLIP interactions play an extremely important role by providing a scaffolding that orients the core CLIP sequence into the peptide-binding groove. The fact that two interacting regions, Ii(103–118) and Ii(81–87), immediately flank the core CLIP sequence may provide a considerable advantage to Ii in competing with partially unfolded polypeptides or short peptides for occupying the peptide-binding site. It is likely that there are many polypeptides available in the ER with sequences that have affinities equal to or greater than that of CLIP for a given class II molecule. We suspect that these flanking interactions may also constrain the register within which CLIP interacts with pockets in the peptide-binding site. However, it remains to be proven that CLIP interacts with all class II molecules in the same register.

The pH dependence of the interactions between DR and the C-terminal fragment, Ii(103–216), suggests that the acidic environment of endosomal vesicles may contribute to the release of Ii from class II αβ. Acidic pH should reduce the overall stability of the αβIi complex through its effects on C-terminal interactions, and it may increase the susceptibility of the C terminus to endopeptidases by releasing it from intimate association with αβ. The C-terminal domain is the site of initial proteolytic cleavage of αβIi (7–10). After release of the C terminus, the αβ-p12Ii complex becomes susceptible to DM-mediated release whether or not there is further proteolytic cleavage on the N-terminal side of CLIP (14, 60–62). It is not known whether the inability of DM to interact with intact αβIi molecules (62) results from steric hindrance mediated by the Ii C terminus or from an indirect effect on the conformation of the class II molecule. In preliminary experiments, we found no evidence that rIi(103–216) inhibits DM-catalyzed peptide dissociation or exchange.

Given the capacity of Ii.BAD-CLIP to associate efficiently with DR1 and direct its transport to endosomal compartments, one must question the role of interactions between CLIP and the peptide-binding groove in Ii function. Our working hypothesis upon initiation of this study was that a major function of Ii is to provide a surrogate peptide (CLIP) to fill the peptide-binding site and stabilize class II molecules during initial folding in the ER and transport to endosomal compartments. Indeed, Zhong et al. (37) demonstrated that binding-site occupancy alone can be sufficient to facilitate the transport of newly synthesized class II molecules in COS cell transfectants. Class II heterodimers with unoccupied binding sites are relatively unstable and prone to aggregation (33–36). However, it has recently been demonstrated that empty IiE molecules are surprisingly stable, with thermal melting transitions greater than many peptide-loaded MHC class I molecules (36). Thus, some class II molecules may have little dependence on binding-site occupancy for folding and transport. However, molecules such as IAl have a clear dependence on Ii for efficient folding and transport (17–19), but the exact role of Ii is unknown. It is possible that interactions outside the peptide-binding groove help to stabilize class II molecules in the ER. An attractive possibility is that occupancy of the peptide-binding site is important but there is no requirement for anchor-pocket interactions. The scaffolding function of Ii may orient CLIP in the peptide-binding site, allowing the network of hydrogen bonds to form between main chain atoms in CLIP and conserved residues in αβ as observed in αβ-peptide crystal structures (50). Non-CLIP interactions with Ii may stabilize this structure even if the binding-site pockets are not occupied by appropriate side chains. In support of this hypothesis, Sadegh-Nas-seri et al. (35) demonstrated that short-lived peptide interactions (presumably lacking stable anchor-pocket interactions) can stabilize empty class II molecules. Non-CLIP interactions may stabilize αβIi, maintaining binding-site occupancy even in class II molecules whose pockets cannot optimally accommodate the anchors available in the CLIP sequence.

It is evident that the CLIP sequence has been selected to play a special role as a promiscuous binder able to interact productively with all class II molecules despite extensive polymorphism in the peptide-binding site. The core sequence contains no side chains at anchor positions that prevent interaction with pockets differing in size, charge, and hydrophobicity through steric hindrance or charge repulsion. Thus charged and large aromatic residues are not present. Conversely, it is unlikely that CLIP can form highly stable complexes with any class II molecule because these amino acids are generally required to interact optimally with dominant pockets in the peptide-binding sites of different class II molecules. We suggest that CLIP has coevolved with class II molecules to be able to bind many class II molecules but none too well. The results of the present study indicate that there is no major detriment to having a CLIP sequence that interacts poorly with pockets in the peptide-binding site. By contrast, it would be extremely detrimental if CLIP bound very stably to a particular class II allele such that it was not efficiently removed in the DM-containing compartments of APC.

Acknowledgments
We thank Dr. Claire-Anne Gutekunst for advice and Joe Moore for excellent technical assistance.

References


