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Surface expression of the invariant chain (CD74) is independent of concomitant expression of major histocompatibility complex class II antigens

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SUMMARY

Whether or not intracellular transport and surface expression of the invariant chain (Ii; CD74) occurs independent of the presence of major histocompatibility complex (MHC) class II molecules was examined by comparing the class II-negative mutant lymphoblastoid cell line 174 × CEM.T2 (T2) and its class II-positive parental cell line 174 × CEM.T1 (T1). We found a similar proportion of Ii being transported to the Golgi complex in T1 and T2, as monitored by the degree of sialic acid addition to glycan side chains of Ii. In agreement with this result, T1 and T2 expressed comparable amounts of Ii at the cell surface, as measured by flow cytometry. This indicates that, although not associated with class II molecules, a proportion of Ii is transported to the plasma membrane. Both in T1 and T2, surface Ii (sIi) was rapidly internalized with a half-life of 3–4 min, suggesting that some Ii enters the endocytic route via the cell surface after being internalized. Our data demonstrate transport of Ii on a route alternative to the endocytic pathway. This alternative route could also account for delivery of newly synthesized class II–Ii complexes to processing compartments in antigen-presenting cells.

INTRODUCTION

The invariant chain (Ii) is known to play a crucial role in the process of major histocompatibility complex (MHC) class II-restricted antigen presentation to autologous T-helper cells.[1] In the human, four different forms of Ii are generated with different efficiencies, both by alternative translational initiation and by differential splicing of a common Ii pre-mRNA: p33, p35, p41 and p43, which are all type II transmembrane glycoproteins.[2,3] The predominant form of Ii, Ii33, has a 30-residue N-terminal cytoplasmic domain, a hydrophobic 26-residue transmembrane region, and a C-terminal extracytoplasmic domain of 160 residues. Ii41 includes an additional exon that is lacking in the corresponding Ii33 form, encoding a cysteine-rich stretch of 64 residues near the C-terminus, homologous to a repetitive sequence of thyroglobulin.[3] Ii35 differs from Ii33 by a N-terminal extension of 16 residues, as a result of alternative translational initiation.[2] This N-terminal extention has been demonstrated to contain an endoplasmic reticulum (ER) retention signal.[4,5] Ii rapidly associates with MHC class II $\alpha\beta$ heterodimers after synthesis in the ER.[6] This association is thought to interfere with the ability of $\alpha\beta$ dimers to bind peptide.[7,8] During transport through the ER and the Golgi complex, both Ii and $\alpha\beta$ dimers undergo post-translational modifications.[9,10] In the *trans*-Golgi the $\alpha\beta$–Ii complexes deviate from the default exocytic pathway to the cell surface. Sorting signals localized to the cytoplasmic tail of Ii target the $\alpha\beta$–Ii complexes to the endosomal compartment,[4,5,11] where they intersect with the endocytic route.[12,13] Here, Ii is detached from class II $\alpha\beta$ dimers by sequential C-terminal proteolytic cleavages performed by acid proteases.[14] As a result, the class II peptide-binding cleft becomes accessible to bind peptide, and class II molecules freed of Ii subsequently emerge on the cell surface.[13,15,16] We have previously demonstrated that Ii is expressed on the plasma membrane of human B-lymphoma cells.[17,18] The surface-expressed forms of Ii have been designated CD74.[19,20] The intracellular pathway that gives rise to Ii surface expression is not elucidated yet. Since Ii is produced in excess over class II α and β chains,[6,9] there is a substantial pool of Ii not associated with class II molecules (free or excess Ii). Free Ii is mainly retained in the ER by formation of mixed trimers, including Ii35 due to its N-terminal ER retention signal,[8,21,22] whereas the class II-associated fraction of Ii appears to be proteolytically removed from $\alpha\beta$ dimers prior to their surface deposition.[23,24] Since the association with class II $\alpha\beta$ dimers is believed to facilitate the egress of Ii from ER,[21,22] we examined whether class II coexpression influences the intracellular transport and surface expression of Ii. Instead of using transfectants we used the mutant cell line

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Abbreviations: ER, endoplasmic reticulum; Ii, invariant chain; MHC, major histocompatibility complex; sIi, cell surface expressed invariant chain.

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174 x CEM.T2 (T2), lacking class II expression due to a genetic deletion in the class II-coding region, and its class II-positive parental line 174 x CEM.T1 (T1).[25] In this report we show that in T1 and T2, Ii displays a similar degree of sialylation indicative of transport to the Golgi apparatus. Both cell lines express comparable amounts of Ii on the cell surface, despite the absence of class II products in T2. In addition, T1 and T2 show the same kinetics of surface Ii (sIi) internalization. Our results suggest an additional role of Ii in delivery of associated class II molecules from the cell surface to processing compartments.

MATERIALS AND METHODS

Cells and reagents

174 x CEM.T1 (T1) and 174 x CEM.T2 (T2) cell lines were kindly provided by P. Cresswell (New Haven, CT).[25] The following monoclonal antibodies (mAb) were used. The Ii (CD74)-specific antibodies BU43, BU45 (kindly provided by I. C. M. MacLennan, Birmingham, UK), and LN2 (Biotest, Dreieich, Germany) have been described previously.[17] The anti-Ii antibody M-B741 (generated by P. Rieber, Munich, Germany) was submitted for CD assignment to the 5th International Workshop on Human Leucocyte Differentiation Antigens (Boston, MA, 1993).[20] BU45 Fab fragments were kindly provided by G. Moldenhauer (Heidelberg, Germany). B7/21 (Becton Dickinson, San Jose, CA), Tü22 (Biotest) and ISCR3[26] react with non-polymorphic epitopes of HLA-DP, -DQ and -DR, respectively. The transferrin receptor-specific mAb Ber-T9 was purchased from Dako (Hamburg, Germany). Fluorescein standard beads were obtained from the Flow Cytometry Standards Corporation (FCSC, Research Triangle Park, NC).

Cell culture

Cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2·5 μg/ml amphotericin B, and cultured at 37° in a humidified 5% CO_2 atmosphere. Brefeldin A (Boehringer Mannheim, Mannheim, Germany) was used at 1 μg/ml.

Metabolic labelling, immunoprecipitation, and two-dimensional SDS–PAGE

T1 and T2 cells were metabolically labelled with [^{35}S]methionine, as described previously.[27] Labelled cells were lysed in 1% Nonidet P-40 (NP-40) and Ii was immunoprecipitated with mAb BU45.[17,20] Two-dimensional separation was performed with non-equilibrated pH-gradient electrophoresis (NEPHGE) as the first dimension, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as the second dimension, as described elsewhere.[27] For neuraminidase treatment, immunoprecipitates were incubated for 180 min at 37° with repeated addition of 10 mU neuraminidase (Bohringer Mannheim) at 0, 30, 60 and 120 min, twice washed in 0·25% NP-40, and subjected to two-dimensional gel electrophoresis.[27]

Flow cytometry

Immunofluorescent staining was performed in polystyrene round-bottomed tubes (Falcon, San Jose, CA). Dilutions and washings were performed throughout in RPMI-1640 medium containing 2% heat-inactivated FCS, 0·1% sodium azide and 10 mM HEPES. Approximately 10^6 cells/sample, suspended in 50 μl of medium, were incubated at 4° with an equal volume of the appropriate dilution of each mAb. After 45 min, cells were washed twice in 500 μl of cold medium, and 2 μg of F(ab')$_2$ goat anti-mouse IgG and IgM fluorescein isothiocyanate (FITC) conjugate (Jackson Immunoresearch, West Grove, PA) was added for 45 min at 4°. Cells were washed again twice and resuspended in 300 μl of medium containing 1 μg/ml propidium iodide (Sigma, St Louis, MO). From each sample the green fluorescence of 10^4 cells was analysed. Dead cells were excluded from analysis by selectively gating on propidium iodide fluorescence, forward and side-scatter parameters. Flow cytometry was performed on a FACScan® cytometer using the LYSYS II® software (Becton Dickinson).

RESULTS

Sialylation of Ii in T1 and T2

The class II-negative B x T hybrid cell line T2 and its class II-positive parental cell line T1 were analysed for intracellular transport of Ii. T2 has a homozygous deletion in the MHC region of chromosome 6, including all functional class II genes.[25] T1 and T2 cells pulse-labelled for 1 hr were immunoprecipitated with anti-Ii mAb BU45. Immunoprecipitates, either treated with neuraminidase (NANAse) or not, were separated by two-dimensional NEPHGE/SDS–PAGE. T1 and T2 exhibited Ii in a typical pattern with numerous acidic modifications (Fig. 1). These modifications were due to sialic acids attached to N- and O-linked glycan side chains,[9,10] indicative of transport to *trans*-Golgi. In T1, class II α and β chains were coprecipitated with BU45 (Fig. 1, T1). Note that the respective spots were absent in T2 (Fig. 1, T2). Sialic acids could be removed by treatment with NANAse at 37° (Fig. 1, Neuraminidase). The increment in the amount of sialylation between T2 and T1 did not exceed 15%, as determined by scanning (data not shown). Thus, it appears that despite the absence of class II products, similar amounts of Ii leave the ER and are transported to the *trans*-Golgi complex in T2 compared to T1.

Ii surface expression on T1 and T2

The amounts of Ii expressed on the surface of T1 and T2 were then determined. Ii surface expression was analysed by flow cytometry using a panel of four different mouse monoclonal antibodies, all recognizing epitopes on the C-terminal (extracytoplasmic) domain of Ii: BU45, BU43, LN2 and M-B741, all clustered to the CD74 panel at the 5th International Workshop on Leukocyte Differentiation Antigens (Boston, MA, 1993).[20] The flow cytometric data presented in Fig. 2 shows that Ii was expressed on both T1 and T2. Comparison with T1 revealed that T2 expressed similar or rather higher amounts of sIi. As a control, only T1 stained positive with anti-HLA-DP, -DQ and -DR mAb, which all gave negative results on T2 as expected. Since T1 and T2 produced comparable amounts of Ii (Fig. 1), surface expression of Ii appears to be independent of the concomitant expression of MHC class II antigens in the T x B hybrid lymphoblastoid cell line T2.

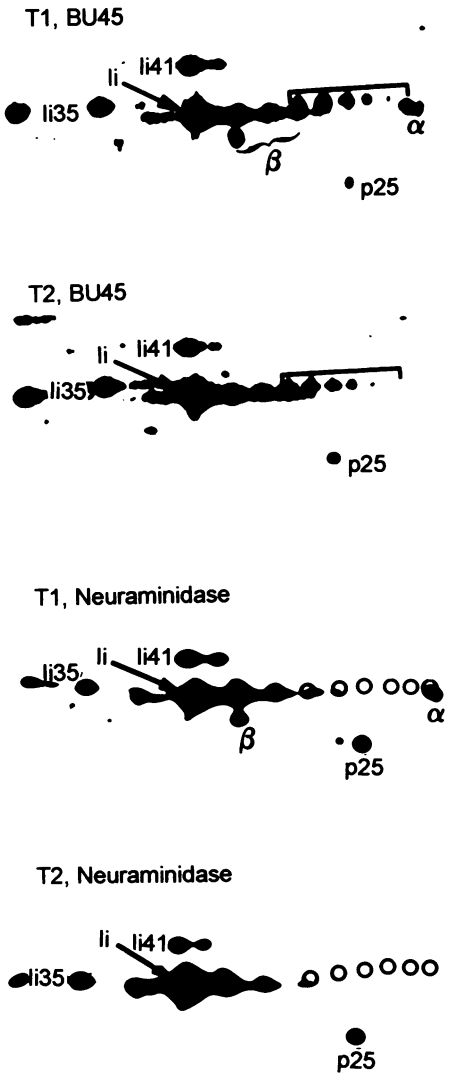

Figure 1. Two-dimensional separation of Ii immunoprecipitates from metabolically labelled T1 and T2 cells pulse-labelled with [^{35}S]methionine for 1 hr. Immunoprecipitates using the anti-Ii mAb BU45 either treated with neuraminidase (Neuraminidase) or not (BU45) were separated in the first dimension by NEPHGE (acidic end to the right side) and in the second dimension by SDS–PAGE. The arrow indicates the major Ii33 high-mannose precursor form of the invariant chain. Sialylated (neuraminidase-sensitive) forms of Ii33 are marked by brackets, and in neuraminidase-treated samples by empty circles. Ii35 and Ii41 indicate the other Ii forms, p25 is an early degradation product of Ii. α and β refer to the chains of MHC class II molecules.

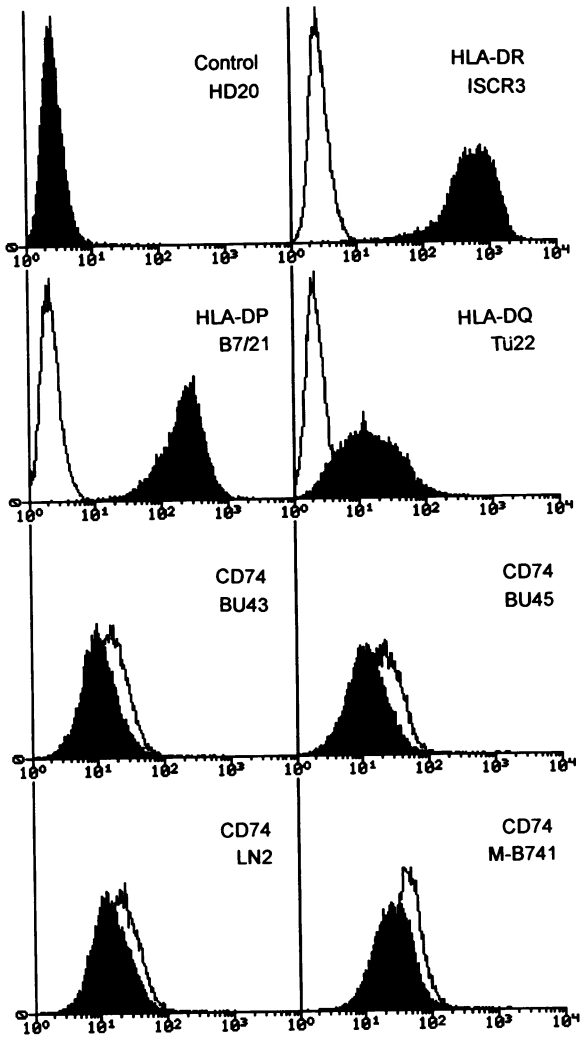

Figure 2. FACS analysis of the class II$^-$/Ii$^+$ T × B lymphoblastoid cell line T2 and its class II$^+$/Ii$^+$ parental cell line T1. T1 (filled histograms) and T2 cells (outlined histograms) were incubated with saturating amounts of anti-HLA-DR mAb ISCR3, anti-HLA-DP mAb B7/21, and anti-HLA-DQ mAb Tü22, each reacting with non-polymorphic epitope of the respective HLA isotype, and the anti-Ii/CD74 mAb BU43, BU45, LN2 and M-B741. The cells were subsequently stained with a FITC-conjugated F(ab')$_2$ goat anti-mouse IgG and IgM as second-step reagent (see the Materials and Methods). Histograms show the relative cell number plotted against the relative intensity of green fluorescence and represent the analysis of 10 000 cells. Both cell lines displayed the same degree of background staining in FACS analysis, as determined with a control mAb HD20 (histograms superimpose).

Surface Ii is rapidly internalized by T1 and T2

As steady-state surface levels of Ii result from the rate of transport to and loss from the cell surface, we compared T1 and T2 for internalization of sIi. T1 and T2 cells were tested for their ability to internalize the Ii-specific mAb BU45 compared to an antibody directed against the transferrin receptor (TfR), Ber-T9. As shown in Fig. 3a, T1 and T2 showed similar kinetics of Ii internalization. Both cell lines rapidly internalized either BU45 monovalent Fab fragments or whole antibody (data not shown), with a half-life of 3–4 min. A similar behaviour was observed for TfR, which is known to undergo rapid internalization and recycling. In contrast, surface expression of HLA-DR on T2 cells was not significantly decreased within 60 min at 37° (data not shown). Internalization of sIi can also be assayed when transport of newly synthesized Ii to the plasma membrane is blocked by treatment with brefeldin A (BFA). This assay circumvents possible effects of tagging sIi with mAb and measures net loss of sIi at steady state.[28] Treatment of T1 and T2 cells with 1 μg/ml BFA at 37° (Fig. 3b) also led to a rapid depletion of sIi. In the presence of BFA, depletion of TfR from the surface was somewhat slower, indicative of a higher degree of recycling relative to Ii. As a control, internalization

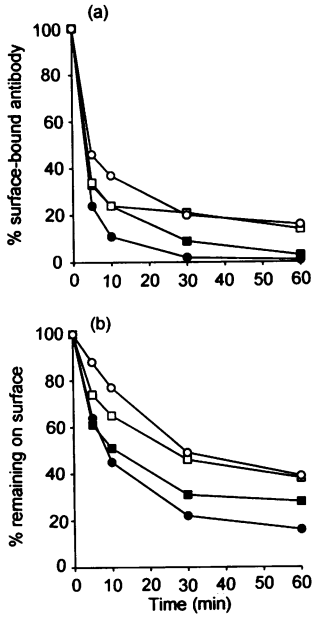

Figure 3. (a) Internalization of prebound CD74/Ii- and CD71/TfR-specific antibodies by T1 and T2. Cells were incubated either with monovalent Fab fragments of anti-Ii mAb BU45 (T1, ■; T2, ●) or the anti-TfR mAb Ber-T9 (T1, □; T2, ○) on ice for 1 hr, washed in cold medium, and were shifted to 37° to allow endocytosis for the indicated periods of time. Subsequently, cells were again placed on ice followed by immunofluorescence staining (see the Materials and Methods). To quantify results, relative fluorescence intensities (median channel values) of cells were converted to mean equivalents of soluble fluorochrome using a fluorescein bead standard. Background staining (< 10%) was likewise quantified and subtracted from the results. Internalization of Ii and TfR is depicted as the percentage of surface-bound mAb remaining after endocytosis at 37° relative to the total amount of mAb bound to cells that were kept on ice throughout (no endocytosis). (b) Loss of Ii and TfR from the cell surface of T1 and T2 at steady state. T1 and T2 cells were treated with 1 μg/ml BFA at 37° for various times. Subsequently, cells were analysed for remaining surface levels of Ii and TfR by FACS analysis using the anti-Ii mAb BU45 (T1, ■; T2, ●) and the anti-TfR mAb Ber-T9 (T1, □; T2, ○). The remaining surface levels of Ii and TfR relative to the respective amounts present on cells not treated with BFA are shown.

assays were performed under hyperosmotic conditions known to block endocytosis. In the presence of 0·45 M sucrose, both the internalization of prebound antibody and the decrease in Ii or TfR surface levels upon BFA treatment were completely inhibited (data not shown). In conclusion, both the low steady-state levels of Ii on the plasma membrane and the short half-life of sIi result from rapid and continuous endocytosis.

DISCUSSION

In this study, we examined the intracellular transport and surface expression of Ii (CD74) in the class II^+/Ii^+ T × B hybrid lymphoblastoid cell line T1 and its class II-negative variant T2, to evaluate the influence of MHC class II coexpression on these processes. We compared the transport of Ii from ER to the *trans*-Golgi in T1 and T2 monitored by the addition of sialic acids to N- and O-linked oligosaccharide side chains. We found no major differences in the amount of sialylation of Ii between T1 and T2 (Fig. 1). Although the majority of Ii remained NANAse-resistant (Fig. 1, arrows), indicating a lack of transport to the medial Golgi, approximately 15–20% of Ii was sialylated after 1 hr of biosynthetic labelling in both cell lines (Fig. 1, brackets). This was an unexpected finding because previous experiments by Marks *et al.*[21] using these cell lines showed that Ii was retained in the ER in trimeric form and failed to be processed in the absence of class II. In that study, anti-class II immunoprecipitates from T1 cells were compared with anti-Ii immunoprecipitates from T2 cells, whereas the present study compared anti-Ii immunoprecipitates from both cell lines.

Transfection studies have revealed ER retention of Ii35 when expressed alone, due to a retention signal within the 16 N-terminal residues of its cytoplasmic tail.[4,5] Furthermore, Ii35 has been shown to mediate ER retention of coexpressed Ii33 by association into mixed trimers in cells lacking class II expression.[5,22] In contrast, Ii33 when not coexpressed with Ii35 localized to vesicular structures.[4,5,11] This localization depended on signals within its 30-residue N-terminal cytoplasmic tail, since a deletion of 15 or more residues resulted in movement to the cell surface.[4,11,29] It was suggested that assembly with class II αβ dimers might mask the ER retention signal of Ii35 and facilitate export of Ii from the ER.[22] In a recent report, we have demonstrated the presence of highly sialylated forms of Ii on the plasma membrane of class II-expressing JOK-1 cells. Not only the most abundant Ii33, but also minor amounts of Ii41 and Ii35, were detected at the cell surface.[30] The results of this study now show that a similar proportion of Ii is exported from the ER in T1 and T2, indicating that the major fraction of Ii that escapes retention can do so irrespective of its association with class II molecules. Since Ii is expressed in large excess over class II molecules, it is conceivable that the fraction of Ii that leaves the ER represents an 'overflow' resulting from the saturation of a limited site of retention in the ER. Assembly of Ii with class II could promote transport of Ii from the ER, but might not be essential.

In a recent study, it was suggested that coexpression of at least one of the HLA class II isotypes (DR, DP, DQ) might be essential for full constitutive CD74 expression.[31] In contrast, our results with the mutant cell line T2 show that a proportion of Ii reaches the cell surface in the absence of class II products. Moreover, the amount of CD74 expressed on T2 is comparable to its parental class II-expressing cell line T1 and to levels of CD74 on normal tonsillar B cells (data not shown),[18] strongly suggesting that trafficking of Ii to the cell surface can occur independent of coexpression of class II molecules. The rapid depletion of Ii from the cell surface upon blocking export of newly synthesized Ii from ER by BFA treatment, and the fact that sIi is largely intact,[18] is indicative of a rapid and immediate route of Ii to the plasma membrane, perhaps the default secretory pathway via *trans*-Golgi. Proteolytic detachment of Ii from class II molecules appears to be required for transport of class II molecules to the cell surface.[23,24] Sorting signals contained in the cytoplasmic tail of Ii have been demonstrated to be responsible for endosomal targeting both of free Ii and class II αβ–Ii complexes.[4,5,11] Thus, Ii surface expression suggests a branched pathway: Ii and class II–Ii complexes might be sorted mainly intracellularly to the endocytic pathway without prior appearance on the cell surface, while part of it

might reach endosomal vesicles after internalization from the cell surface. Consistent with this, Harter & Mellman[32] observed limited surface expression of the lysosomal membrane protein Igp-A when expressed at high levels, which at low expression is sorted intracellularly.

Our data demonstrating rapid internalization of sIi in both T1 and T2 confirm previous results by Roche *et al.*[28] with the B-lymphoblastoid cell line .45 and its class II-negative variant .174. This study showed that rapid internalization of HLA-DR $\alpha\beta$–Ii complexes required the Ii cytoplasmic tail. A recent report provided evidence for at least two sorting signals, in the cytoplasmic tail of Ii.[33] One, referred to as the di-leucine motif, was previously shown to be responsible for both endocytosis and endosomal/lysosomal targeting.[34] Thus, surface-expressed Ii clearly has the ability to mediate rapid internalization of associated class II $\alpha\beta$ dimers. We have previously shown that a proportion of sIi is associated with class II molecules on the B-cell line JOK-1.[18] Both soluble and plasma membrane-associated mutant Ii were demonstrated to inhibit peptide binding at the cell surface.[8,29] HLA-DR $\alpha\beta$ dimers within $\alpha\beta$–Ii complexes are devoid of peptides *in vivo*,[35] indicating that association with Ii, both intracellularly and at the plasma membrane, interferes with the peptide-binding ability of class II molecules.[7,15,29] Since Ii is transported to the plasma membrane in cells lacking class II molecules, as shown with T2, it is tempting to speculate that free Ii also present at the cell surface of class II-positive cells may associate with 'empty' $\alpha\beta$ dimers that have lost low-affinity bound peptides, thereby promoting their endocytosis and delivery to processing compartments. Upon internalization, Ii dissociates from class II $\alpha\beta$ dimers,[28] which subsequently may reload peptide in the endocytic pathway and present it after recycling to the plasma membrane.[36]

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