A three-amino-acid-long HLA-DRβ cytoplasmic tail is sufficient to overcome ER retention of invariant-chain p35

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Accepted 19 July 2005
Journal of Cell Science 118, 4679-4687 Published by The Company of Biologists 2005
doi:10.1242/jcs.02592

Summary

The p35 isoform of the human invariant chain (Iip35) contains an N-terminal RXR endoplasmic-reticulum (ER) retention signal that becomes nonfunctional only after assembly with MHC-class-II molecules. We have previously shown that the MHC-class-II β-chain cytoplasmic tail is crucial for the maturation of class-II/Iip35 complexes. In order to shed some light on the molecular determinants involved in shielding the RXR motif, we performed site-directed mutagenesis of the DRβ chain and Ii cytoplasmic domains. Chimeric β chains with irrelevant cytoplasmic tails allowed the efficient transport of Iip35 out of the ER in transiently transfected HEK 293T cells. An alanine scan of the cytoplasmic tail of HLA-DRβ confirmed that no specific motif is required to overcome ER retention. Surprisingly, a β chain with a three-amino-acid-long cytoplasmic tail (Tyr-Phe-Arg) was sufficient to overcome the Iip35 RXR motif. Moreover, replacement of residues F231 and R232 with alanines created a cytoplasmic tail (Tyr-Ala-Ala) that allowed ER egress. Given the limited length of this tail, steric hindrance would only be possible if the Ii ER retention motif was close to the membrane in the first place. However, this is not likely because an Ii molecule with an internal cytoplasmic deletion bringing the RXR motif closer to the membrane is not retained in the ER, even in the absence of class-II molecules. These results suggest that MHC-class-II molecules overcome ER retention and prevent COPI binding to the Iip35 RXR motif through a mechanism distinct from steric hindrance by its β chain.

Key words: HLA, Iip35, RXR, Di-arginine, Antigen presentation

Introduction

The major histocompatibility complex (MHC) class-II molecules are expressed on antigen-presenting cells of the immune system and display foreign antigenic peptides to T cells (Cresswell, 1994). During their assembly in the endoplasmic reticulum (ER), class-II αβ dimers associate with preformed trimers of the invariant chain (Ii) to form nonameric $(\alpha\beta Ii)_3$ oligomers (Marks et al., 1990; Roche et al., 1991; Lamb and Cresswell, 1992). Ii folds in part through the peptide-binding groove and promotes the folding of class-II molecules by preventing aggregation and premature binding of endogenously synthesized peptides (Roche and Cresswell, 1990; Romagnoli and Germain, 1994; Stumptner and Benaroch, 1997; Busch et al., 1996; Anderson and Miller, 1992; Hitzel and Koch, 1996). Targeting of the complex to the endocytic pathway is mediated by two leucine-based motifs in the cytoplasmic tail of Ii (Pieters et al., 1993; Odorizzi et al., 1994). When these endosomal localization signals are obliterated, Ii is found mostly at the cell surface and has a much longer half-life (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Anderson et al., 1993; Roche et al., 1993; Nijenhuis et al., 1994). Within acidic compartments, the Ii lumenal domain is progressively degraded facilitating dissociation from MHC-class-II molecule and peptide loading (Ericson et al., 1994; Jensen et al., 1999; Thery et al., 1998).

The human gene encoding the invariant chain produces two separate mRNAs that differ by an alternatively spliced exon. Furthermore, the alternative use of two in-phase start codons on each mRNA produce either the major p33 and p35 or the minor p41 and p43 isoforms (Strubin et al., 1986; O'Sullivan et al., 1987). Iip35 differs from p33 by an N-terminal cytoplasmic extension of 16 amino acids containing a strong di-arginine (RXR) ER retention motif (Schutze et al., 1994). This signal plays a crucial role in the proper ER localization of many transmembrane proteins and regulates the association of multimeric protein complexes (Jackson et al., 1990; Pelham, 1995). By virtue of a COPI-mediated retrograde transport mechanism, the RXR motif usually mediates retrieval of unassembled subunits from post-ER compartments of the exocytic pathway (Cosson and Letourneur, 1994; Zerangue et al., 1999; Margeta-Mitrovic et al., 2000; Bichet et al., 2000; Standley et al., 2000; O'Kelly et al., 2002; Yuan et al., 2003). Compared with their p35 counterpart, Iip33 homotrimers are sorted to the endocytic pathway in the absence of class-II molecules (Lamb and Cresswell, 1992; Bakke and Dobberstein, 1990; Teyton et al., 1990). Accordingly, mixed trimers consisting of p33 and at least one p35 molecule accumulate in the ER (Lamb and Cresswell, 1992; Marks et al., 1990). In the presence of class-II molecules, the RXR motif of Iip35 is

inactivated (Marks et al., 1990). Still, the intracellular route to the endosomes presumably differs between p35-including nonamers and $(\alpha\beta Iip33)_3$, because the former complexes are not detected at the cell surface (Warmerdam et al., 1996). In normal B cells, the proportion of the Ii pool bearing this ER retention signal has been estimated to be 20% (Anderson et al., 1999). Therefore, Iip35 would play an important role in coordinating the assembly and transport of newly synthesized Ii and class-II molecules.

It has been reported that a serine residue (S8) present exclusively in the p35 cytoplasmic tail is phosphorylated in B-cell lines and transfected HeLa cells (Anderson et al., 1999; Anderson and Roche, 1998; Kuwana et al., 1998). Although the exact trafficking step requiring such phosphorylation is still a matter of debate, it is clearly a prerequisite for ER egress and efficient sorting of p35/class-II complexes to the endocytic pathway (Anderson et al., 1999; Anderson and Roche, 1998; Kuwana et al., 1998).

Recently, O'Kelly and co-workers suggested that the binding of 14-3-3 and βCOP is mutually exclusive on Iip35 (O'Kelly et al., 2002). The 14-3-3 family members are highly acidic dimeric cytoplasmic proteins that bind to phosphoserine motifs (Muslin and Xing, 2000; Schutze et al., 1994; Tzivion et al., 2001; van Hemert et al., 2001; Yaffe, 2002). It was concluded that the 14-3-3 binding site constitutes a 'release' motif allowing ER egress. However, this model does not take into account the fact that, even when associated with a 14-3-3 protein, Iip35 will not leave the ER in the absence of class-II molecules (Marks et al., 1990; Kuwana et al., 1998; Khalil et al., 2003).

As a first step in characterizing the peculiar intracellular trafficking of Iip35, we have recently shown that the cytoplasmic tail of the class II β chain is crucial for overcoming the ER-retention motif (Khalil et al., 2003). Our results demonstrated that a tailless DR molecule is expressed at the plasma membrane of transfected cells only in the absence of Iip35. Accordingly, Iip35 leaves the ER and is sorted to the endocytic pathway only when co-transfected with class-II molecules presenting a wild-type β chain. Here, we refined the mapping of the important structural determinant and conclude that a three-amino-acid cytoplasmic β tail is sufficient to allow full egress of Iip35.

Materials and Methods

cDNAs and mutagenesis

cDNAs encoding the p33 and p35 isoforms of the human invariant chain (Sekaly et al., 1986) and HLA-DRβ 0101 (Tonnelle et al., 1985) were obtained from E. O. Long (NIH, Bethesda, MA). The cDNA coding for the truncated DRα (αTM) and DRβ (βTM) chains have been described previously (Robadey et al., 1997; Khalil et al., 2003). The cytoplasmic tail of HLA-DRβ was replaced by that of either a HLA-DMβ variant (βcytoDM mut) (Deshaies et al., 2005) or HLA-DOβ (Brunet et al., 2000). The cDNA encoding the p35 isoform with no endosomal sorting signals [pREP4 Iip35 leucine, isoleucine/methionine, leucine (LI/ML)] has been described previously (Khalil et al., 2003). The alanine scan of HLA-DRβ cytoplasmic tail was done by PCR overlap extension (Ho et al., 1989). The sequences of the mutagenic oligonucleotides are available upon request. The cytoplasmic tail of HLA-DRβ was replaced by segments of different length and composition by cloning overlapping oligonucleotides. An intermediate DRβ molecule (DRβ AAA.1) had to be constructed to facilitate cloning. This mutation creates an *Avr*II restriction site near the stop codon. The overlapping oligonucleotides were cloned into *Sty*I and *Avr*II sites of pBS KS 3′DRβAAA 1.3. The chimeric cDNA was cloned as a *Bam*HI fragment into pBUD-CE4 (Invitrogen). The cDNA encoding Iip35 with amino acids 17-45 deleted (Iip35 Δ17-45) was constructed by deleting the p33 cytoplasmic region from Iip35 by PCR overlap extension splicing (Horton et al., 1989). This deleted cDNA was cloned into the pREP4 vector (pREP4 Iip35 Δ17-45). The cDNA encoding a mutant Ii devoid of most of its cytoplasmic tail (Iip35 Δ2-35 or Iip33 Δ20) was obtained from O. Bakke (University of Oslo, Norway) (Roche et al., 1992). Mutation of the cytoplasmic prolines (Iip35 P31A and Iip35 P37A,P40A) was done by PCR overlap in the pREP4 Iip35 LI/ML vector.

Cell lines and transfections

MHC-class-II-negative HEK 293T (a kind gift from E. Cohen, University of Montreal, Quebec, Canada) and HeLa (ATCC CCL-2) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wisent, St-Bruno, Quebec, Canada) containing 10% foetal bovine serum (FBS; Wisent). For transient expression in HEK 293T, cells were co-transfected by the calcium-phosphate-precipitation method using 2 μg each DNA (Brunet et al., 2000). Cells were analysed 2-3 days after transfection. Stable transfection of the mutated class-II molecules in HeLa cells was performed using Fugene6 (Roche). Cells resistant to 100 μg ml^{-1} Zeocin™ (Cayla, Toulouse, France) and expressing class-II αβ heterodimers were sorted using the L243 monoclonal antibody (mAb) coupled to magnetic beads (Dynal, New York, NY, USA). The Iip35 LI/ML cDNA was then transfected using Fugene6 and selected in 50 U ml^{-1} hygromycin (Cederlane, Hornby, BC, Canada).

Antibodies

BU45 (IgG_1) is a mAb specific to the C-terminal portion of the human Ii (Wraight et al., 1990). ISCR3, a kind gift from R. Busch (Stanford University, CA), recognizes the αβ heterodimer through an epitope on the DRα chain (Watanabe et al., 1983). Goat anti-mouse antibody coupled to Alexa Fluor® 488 was obtained from Molecular Probes (Eugene, OR, USA).

Flow cytometry

For surface staining of Ii, cells were stained using Bu45 followed by a goat anti-mouse IgG coupled to Alexa Fluor 488. Intracellular staining for class-II molecules or Ii was done on saponin-permeabilized cells as previously described (Brunet et al., 2000; Khalil et al., 2003). Briefly, cells were washed, fixed in 4% paraformaldehyde, permeabilized with saponin (Brunet et al., 2000) and stained for Ii or class-II molecules using BU45 or ISCR3, respectively. Cells were analysed on a FACScalibur® flow cytometer (Becton Dickinson, Mississauga, ON, Canada).

Results

Chimeric class-II molecules with irrelevant cytoplasmic tails mask the Iip35 RXR motif

The Iip35 RXR retention signal can be masked by the β-chain cytoplasmic tail of all three human MHC-class-II isotypes (HLA-DR, HLA-DP and HLA-DQ) (Khalil et al., 2003). Here, we investigate whether this region of HLA-DR encompasses a specific signal required to overcome the Iip35 retention motif. We have recently developed a transient expression system in class-II^- Ii^- HEK 293T cells in order rapidly to assess the capacity of any class-II molecule (whether wild type or mutant)

to mask the Iip35 RXR sequence (Khalil et al., 2003). By using a mutant Iip35 devoid of its two leucine-based lysosomal sorting signals (Iip35 LI/ML), one can easily and directly monitor ER egress by flow cytometry. Indeed, this modification directs Ii/class-II complexes to the plasma membrane rather than to the endocytic pathway, allowing cell-surface staining with the Ii-specific mAb BU45. Having shown that the DRα cytoplasmic tail was dispensable for the masking of the Iip35 retention signal, we have used throughout this study a tailless chain truncated after Gly-216 (Robadey et al., 1997). First, as controls, DR αTM/β (truncated α chain, wild-type β chain) or DR αTM/βTM were expressed in HEK 293T cells along with Iip35 LI/ML. Cells were analysed by flow cytometry 48 hours after transfection (Fig. 1A,B). As expected, despite similar total levels of class-II molecules, we observed the plasma-membrane expression of Iip35 LI/ML on cells transfected with αTM-β but not on those expressing αTM-βTM (Fig. 1B). This result is not due to a gross conformation defect of the truncated molecules, because we have previously shown that the DR αTM-βTM class II is well expressed at the cell surface in the absence of Iip35 and is recognized by conformational antibodies (Khalil et al., 2003). We took into account the transfection efficiency of Ii by expressing the results as a ratio of the mean fluorescence value obtained for cell surface BU45 mAb staining to that obtained following permeabilization (Fig. 1C). To investigate the presence of a putative signal in the HLA-DRβ cytoplasmic tail, we used a chimeric molecule in which almost the entire cytoplasmic region was replaced by the one of the non-classical MHC class-II molecules HLA-DO (βcytoDO) or by an irrelevant tail produced by a frame shift in the HLA-DM C-terminus coding region (βcytoDMmut) (Fig. 1A). These chains were expressed transiently in HEK 293T cells together with the truncated DRα chain (αTM/βcytoDMmut and αTM/βcytoDO) and Iip35 LI/ML. Fig. 1B shows the clear surface expression of Iip35 in the fraction of transfected cells expressing either β chain. Similar results were obtained for stably transfected HeLa cells (data not shown). The ratios between surface and total levels of Iip35 indicate that both cytoplasmic tails can efficiently overcome the RXR retention motif (Fig. 1C).

Ability of the DRβ cytoplasmic region to overcome the RXR motif of Iip35 is sequence independent

Fig. 2 shows a sequence alignment of the class-II β chain cytoplasmic sequences that have been shown so far to

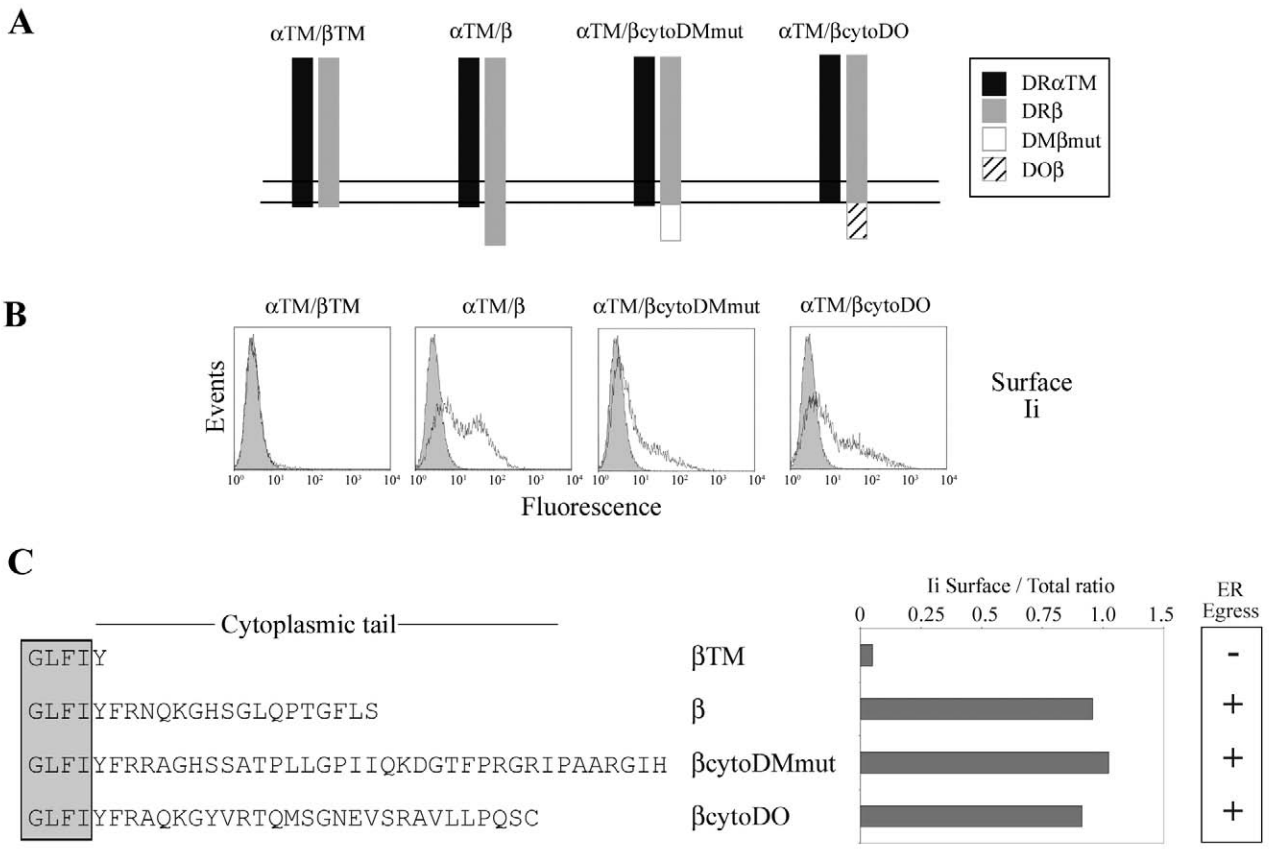

Fig. 1. Irrelevant cytoplasmic tails on DRβ overcome the Iip35 ER retention motif. (A) Schematic representation of the chimeric class II α and β chain combinations expressed in HEK 293T cells. (B) HEK 293T cells were transiently co-transfected with cDNAs for the indicated chimeric molecules and Iip35 LI/ML. After 48 hours, cells were analysed for Ii expression by flow cytometry using BU45 either before (surface) or after (total) permeabilization with saponin. (C) The ratios of surface to total Ii are shown next to the C-terminal amino-acid sequences of the chimeric molecules' cytoplasmic tails. Class-II expression was measured on permeabilized cells using the ISCR3 mAb. The mean fluorescence values (MFVs) for DR αTM/β, DR αTM/β, DR αTM/βcytoDMmut and DR αTM/βcytoDO expression were 280, 267, 275 and 251, respectively. The MFV for untransfected cells was 4 (data not shown).

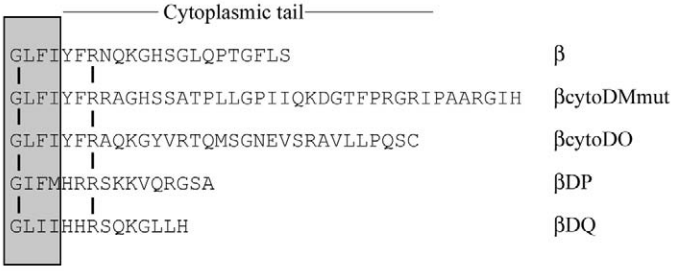

Fig. 2. Sequence alignment of the cytoplasmic tails of the MHC-class-II molecules β chains. The C-terminal amino-acid sequence of the classical MHC-class-II molecules DRβ, DPβ and DQβ are shown along with the chimeric molecules DRβcytoDMmut and DRβcytoDO. The conserved Arg232 is indicated.

overcome ER retention of Ii. A single amino acid (R232) is conserved between all these tails. To verify the importance of this residue and definitely to rule out the need for a specific more-C-terminal sequence, we performed an alanine scan on this region (Fig. 3). Mutant DRβ chains were expressed transiently in HEK 293T cells together with the truncated DRα chain and Iip35 LI/ML. Class-II levels were comparable and based on the ratio of surface to total invariant chain expression, we conclude that all mutants were able to overcome the RXR motif. Even the R232A mutation allowed ER egress and cell-surface expression of Iip35 LI-ML.

Three cytoplasmic DRβ amino acids are sufficient to overcome retention

We next investigated the minimal requirements, in terms of length, for the HLA-DRβ cytoplasmic tail to overcome the Ii retention motif. Stop codons were introduced by site-directed mutagenesis at various positions in the C-terminal coding region of DRβ. First, the non-potent βTM tail was extended by one or two amino acids to generate βYF and βYFR (Fig. 4A). These DRβ chains were transiently expressed in HEK 293T cells together with the DRαTM and Iip35 LI/ML. The ratios of surface to total Ii demonstrate that, whereas the truncated βYF molecule does not allow ER egress of Ii, extending the DRβ chain by only one additional residue (βYFR) is sufficient fully to overcome the RXR retention motif. Interestingly, substitution of the Phe and Arg residues for alanines (βYAA) also allowed egress of Iip35. However, in accordance with the results presented above, we found that a cytoplasmic tail extension of only one residue beyond the transmembrane-lining tyrosine (βYA) is not sufficient to overcome ER retention. The capacity of the βYFR and βYAA tails to chaperone Iip35 out of the ER was confirmed in stably transfected HeLa cells (Fig. 4B). Altogether, these results demonstrate that a three-amino-acid cytoplasmic tail is sufficient to overcome the Iip35 RXR motif.

Replacing Tyr230 with Arg partially restores function

Given the role of the membrane-proximal residues in the capacity to overcome the Ii-retention motif, we investigated the importance of Tyr230 at the junction between the transmembrane and cytoplasmic regions of DRβ. Tyrosine residues delimiting transmembrane domains usually span the interfacial region of the phospholipid bilayer and are not fully exposed in the cytoplasm (Bowie, 2005). We generated a truncated mutant DRβ chain with a positively charged C-terminal Arg230 (DRβ R) (Fig. 5). When this mutant was co-transfected in HEK 293T cells together with a truncated α chain and Iip35 LI/ML, we were able to detect cell-surface expression of the invariant chain. However, the surface over total Ii ratio showed that, in comparison to the wild-type DRβ chain, DRβ R was less potent. The fact that a single Arg residue somehow overcomes the Iip35 ER retention motif strongly argues against a model where the DRβ chain operates through steric hindrance masking.

Position-dependent activity of the RXR motif

The above results, demonstrating that three membrane-proximal residues are sufficient fully to overcome ER retention of Iip35, are difficult to reconcile with a possible interaction between the DRβ tail and the RXR motif. However, we cannot exclude the possibility that the N-terminal region of Iip35 folds back close to the membrane and that the RXR motif lies next to the key residues identified in DRβ. This would imply that the fine conformation of the Iip35 cytoplasmic tail is crucial for positioning the RXR sequence in the vicinity of DRβ. To test this hypothesis, we disrupted the secondary conformation of the Ii tail by replacing three proline residues with alanine (Fig. 6). By analogy, mutation of proline 31 (position 15 in Iip33) next to the endosomal sorting signal was shown to prevent bending of the cytoplasmic helix and to abolish internalization of Ii (Motta et al., 1995). As shown in Fig. 6B, mutation of Iip35 LI/ML prolines at position 31 (P31A) or 37 and 40 (P37A,P40A) did not affect the capacity of the RXR sequence to retain Iip35 molecules in the absence of class II molecules (Fig. 6B, black bars). When class-II molecules (DR αTM/β) were co-transfected with these mutants, an efficient

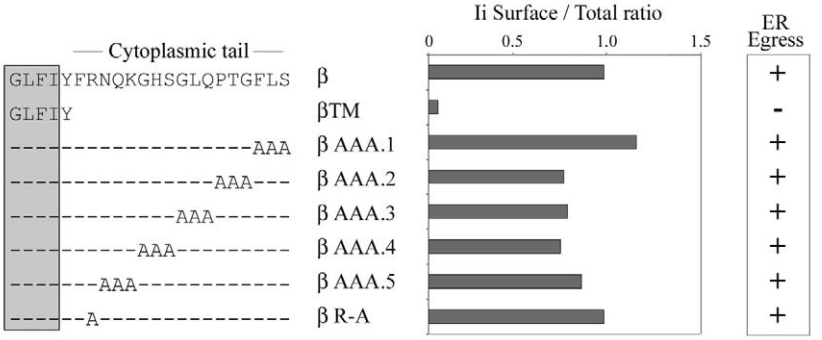

Fig. 3. Alanine scan of DRβ cytoplasmic tail. HEK 293T cells were transiently co-transfected with the indicated mutant molecules and with pREP4 Iip35 LI/ML and tested after 48 hours. Cells were analysed by flow cytometry using BU45 either before (surface Ii) or after (total Ii) permeabilization with saponin. The ratios of surface to total Ii are shown. The total ISCR3 mean fluorescence value (MFV) for all the chimeric class-II molecules was between 242 and 260. The MFV for the secondary antibody alone was 5 (data not shown).

masking of the motif was observed (Fig. 6B, grey bars). These results argue against a strict interaction between the membrane-proximal DRβ residues and specific regions of Iip35.

The possibility remains that the RXR motif is located near the transmembrane region, independent of the prolines. Also, the nuclear magnetic resonance structure of peptides corresponding to the Ii cytoplasmic tail showed the formation of a co-planar triple helix (Motta et al., 1997). We tested this hypothesis by deleting the p33 cytoplasmic tail region, thereby bringing the Ii N-terminal region and the RXR motif next to the transmembrane region (Iip35 Δ17-45). However, as shown in Fig. 7, deletion of this region inactivated the Iip35 retention motif and allowed egress from the ER even in the absence of MHC-class-II molecules. Cell-surface expression of Iip35 Δ17-45 in the absence of MHC-class-II molecules was comparable to the level of expression of a mutant Ii molecule devoid of most of its cytoplasmic tail (Iip35 Δ2-35). This indicates that the retention motif was completely inactive in Iip35 Δ17-45, in line with the need for a 'comfort zone' between the RXR sequence and the membrane. This result suggests that the RXR motif is not close to the membrane, at least in the absence of class-II molecules, and would probably not be subjected to steric hindrance by the short tails (βYAA, βYFR, βR) capable of restoring ER egress.

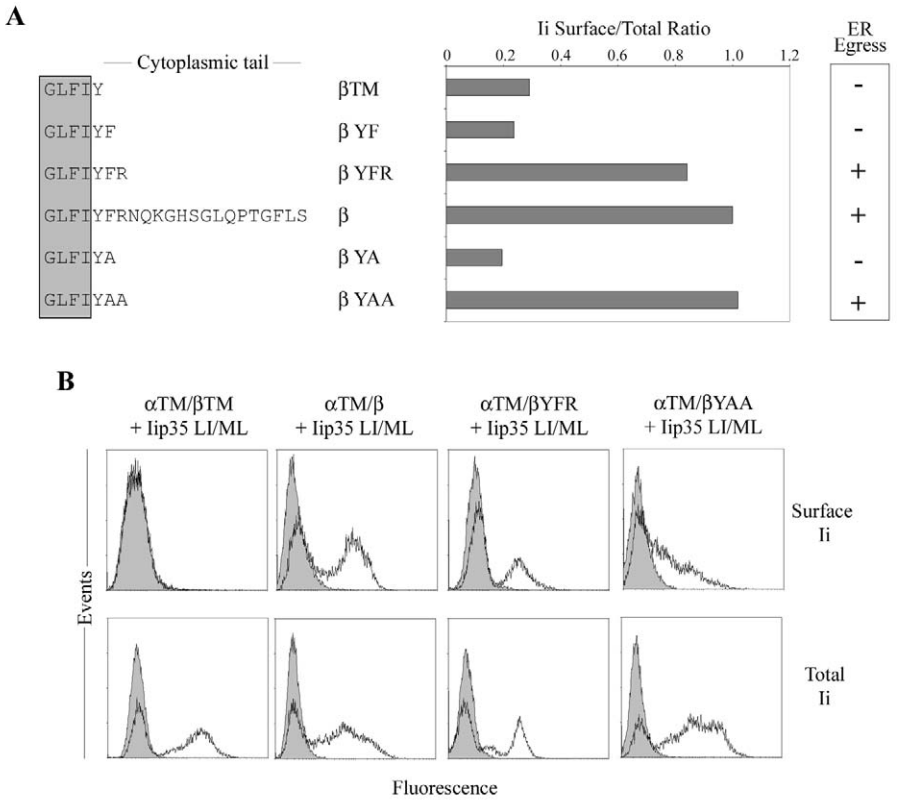

Fig. 4. Truncation of DRβ cytoplasmic tail beyond Arg232 hinders ER egress. (A) HEK 293T cells were transiently co-transfected with the indicated chimeric molecules and with pREP4 Iip35 LI/ML and tested after 48 hours. Cells were analysed by flow cytometry using BU45 either before (surface Ii) or after (total Ii) permeabilization with saponin. The ratios of surface to total Ii are shown. The total ISCR3 mean fluorescence value (MFV) for all the chimeric class II molecules was between 223 and 248. The MFV for the secondary antibody alone was 5 (data not shown). (B) Hela cells stably expressing the indicated class II molcules were supertransfected with Iip35 LI/ML cDNA and stained for Ii using BU45.

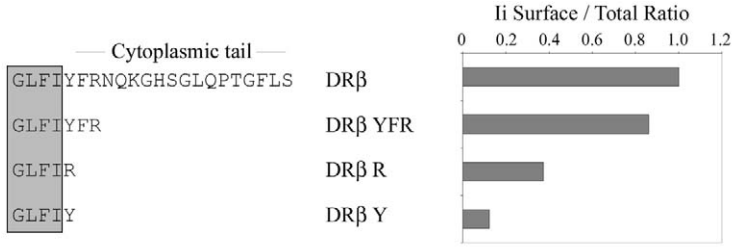

Fig. 5. Replacement of Tyr230 by an Arg in a truncated DRβ molecule partially restores Iip35 egress. HEK 293T cells were transiently co-transfected with the indicated chimeric molecules and with pREP4 Iip35 LI/ML and tested after 48 hours. Cells were analysed by flow cytometry using BU45 either before (surface Ii) or after (total Ii) permeabilization with saponin. The ratios of surface to total Ii are shown. The total ISCR3 mean fluorescence value (MFV) for all the chimeric class II molecules was between 235 and 255. The MFV for the secondary antibody alone was 5 (data not shown).

Discussion

The chaperone role of Ii in MHC-class-II folding and trafficking has been well characterized (Cresswell, 1994). The reciprocal importance of class-II molecules for Ii maturation is also documented (Kuwana et al., 1998; Marks et al., 1990). Recently, we have shed some light on the mechanism by which DR overcomes the Iip35 RXR motif. Our results demonstrated that the cytoplasmic region of the β chain is crucial for the Ii/class-II complex to leave the ER (Khalil et al., 2003).

Although RXR or KKXX ER retention motifs have been described in several oligomeric proteins, the mechanisms by which they are masked remain ill defined. In the case of the human high-affinity receptor for IgE (FcεRI), Letourneur and Cosson have shown that, upon formation of the complex in the ER, the cytoplasmic tail of the γ subunit masks the α chain dilysine motif (Letourneur et al., 1995). Using various chimeric constructs and reporter molecules, they demonstrated that any cytoplasmic domain longer than 15 amino acids on the γ subunit successfully hides the α chain KK motif and allows plasma-membrane expression. These results led to the conclusion that the masking is caused by steric hindrance following juxtaposition of the two cytoplasmic regions.

In the case of DRβ, we showed that the presence of only

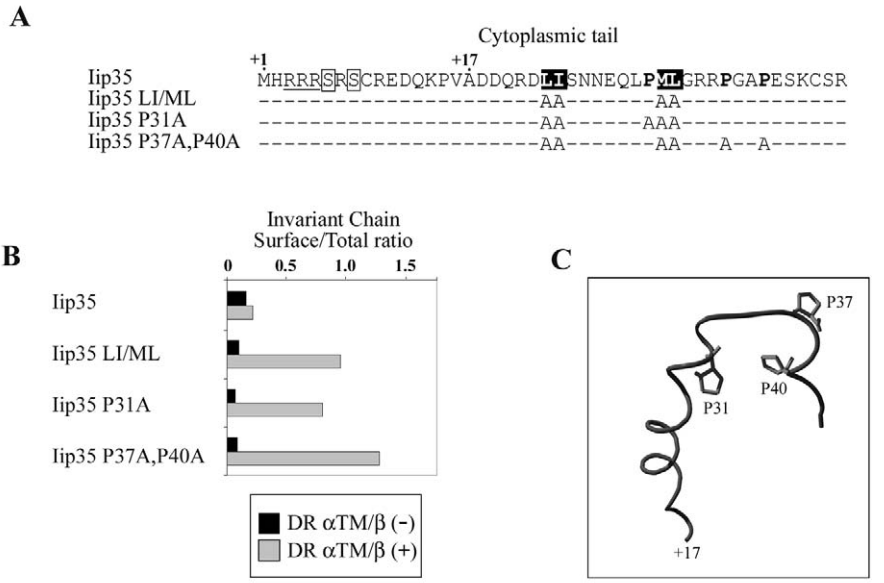

Fig. 6. Proline residues in the Iip35 cytoplasmic tail are not required for function of the RXR motif and its masking by HLA-DR. (A) N-Terminal amino acid sequence of the Iip35 proline mutants. Positions 1 and 17 are the initiation sites of Iip35 and Iip33, respectively. The arginine residues in the RXR motif are underlined, the leucine-based targeting signals are in black boxes, prolines in Iip33 region are in bold, and Ser6 and Ser8 are boxed. (B) HEK 293T cells were transiently co-transfected with the indicated invariant-chain molecules and with or without DR αTM/β and tested after 48 hours. Cells were analysed by flow cytometry using BU45 either before (surface Ii) or after (total Ii) permeabilization with saponin. The ratios of surface to total Ii are shown. (C) Schematic representation of the p33 cytoplasmic tail structure as determined by nuclear magnetic resonance (Motta et al., 1997). Proline residues that were mutated to alanine are indicated.

three cytoplasmic amino acids, the nature of which does not appear to be crucial, allows ER egress of Iip35. For three main reasons, these data are difficult to reconcile with a trivial steric hindrance mechanism in which DR prevents access of COPI to the Iip35 cytoplasmic tail. First, the RXR motif is located 41 residues away from the Ii transmembrane-cytoplasmic domain junction and would probably not be close enough to the membrane to be influenced by a three-amino-acids tail. Second, it would be difficult to ascribe steric masking properties to a three-residue DRβ tail given that the closely juxtaposed DRα cytoplasmic tail, comprising 15 amino acids with complex side chains, does not impinge on ER retention. Finally, even the full-length DRβ cytoplasmic tail does not overcome ER retention in the presence of an unphosphorylated Iip35.

Other recent studies have proposed the existence of key amino-acid sequences capable of overcoming RXR motifs on binding partners. Results on the NMDA receptor showed that a cytoplasmic four-amino-acid stretch next to the membrane in NR2 regulates the export of associated RXR-bearing NR1 subunits. Because site-directed mutagenesis in this NR2 motif abrogated the activity, the results suggested that certain amino-acid sequences could allow ER egress independently of their capacity sterically to mask retention signals (Hawkins et al., 2004). Very similar results were obtained using the kainate receptor GluR6a and GluR5c subunits (Jaskolski et al., 2004). The mechanisms of action of such 'release' motifs have not yet been characterized. For Ii, the need for a precise DRβ amino acid sequence suggests that facilitation of ER export is mediated by a mechanism different from those involving, for example, DXE or FCYENE motifs (Nishimura and Balch, 1997; Ma et al., 2001).

In addition to dominant trans-acting motifs, it was reported that cis-acting sequences can play a role in the inactivation of RXR retention signals. Although the ligand is unknown, a PDZ-binding domain in an alternatively spliced NR1 isoform was shown to inactivate the closely positioned RXR retention motif upstream in the cytoplasmic region (Scott et al., 2001). Also, protein-kinase-C phosphorylation of serine residues next to the RXR motif in the NR1 subunit of the NMDA receptor

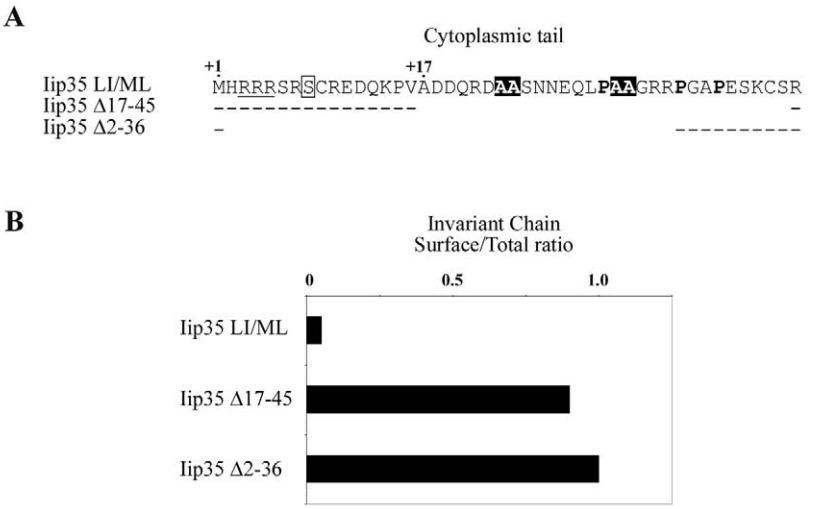

Fig. 7. The RXR motif is inactive when close to the membrane. (A) N-Terminal amino-acid sequence of Iip35 LI/ML and the mutants with deletions in this region. Positions 1 and 17 are the initiation sites of Iip35 and Iip33, respectively. The arginine residues in the RXR motif are underlined, the original position of the mutated leucine-based targeting signals are in black boxes, prolines in Iip33 region are in bold and the phosphorylated Ser8 is boxed. (B) The indicated invariant-chain molecules were transiently co-transfected in HEK 293T cells and tested after 48 hours. Cells were analysed by flow cytometry using BU45 either before (surface Ii) or after (total Ii) permeabilization with saponin. The ratios of surface to total Ii are shown.

was suggested to promote ER egress (Scott et al., 2001). O'Kelly and collaborators recently suggested the existence of a cis-acting release motif on Iip35 (O'Kelly et al., 2002). It was postulated that the binding of 14-3-3 proteins to the phosphorylated Ser8 would prevent the interaction with β-COP and allow ER egress. Although its exact role remains obscure, Ser8 is clearly part of a release motif because its mutation to alanine prevents egress even in the presence of wild-type HLA-DR (Kuwana et al., 1998). In light of our results, it is possible that the membrane-lining residues in the class II β-chain cytoplasmic tail also represent a release motif or domain.

The YFR sequence of DRβ is replaced by HRR in DPβ and HHR in DQβ, two other isotypes that overcome the retention motif of Iip35. Despite the presence of a conserved Arg, our mutagenesis results suggest that this residue is not crucial. Interestingly, a single Arg residue at the C-terminus of a truncated chain (Fig. 5, β R) can somehow overcome the retention motif. The possibility remains that this Arg residue corrects a defect in the membrane insertion of the DR βTM chain with no positively charged residue at its C-terminal end. However, the fully truncated DR αTM-βTM heterodimer is found at the cell surface in the absence of Iip35, suggesting that its conformation is adequate (Khalil et al., 2003). Also, the DR βYAA does not include any charged cytoplasmic residue and efficiently overcomes the RXR motif of Iip35 (Fig. 4). Rather, the arginine might 'snorkel' and position its long apolar side chain through the hydrocarbon region of the membrane to expose only its polar extremity to the interfacial region (Killian and von Heijne, 2000). This would artificially extend the transmembrane region and could be in line with a steric effect. Although we propose that DR does not mask the RXR motif directly by steric hindrance, we cannot rule out the possibility that the β chain cytoplasmic tail imposes structural constraints on the folding of the Ii cytoplasmic tail. The DR βYFR residues are found at the membrane-water interfacial region and this location could determine their precise orientation (Granseth et al., 2005). The class-II and Ii molecules interact in their lumenal part but also at the level of their transmembrane regions (Castellino et al., 2001; Ashman and Miller, 1999). Owing to such close proximity in the nonameric complex, it is possible that the three membrane-proximal DRβ residues (YFR) affect the whole Ii cytoplasmic region and indirectly bring the RXR motif near the membrane, out of reach for COPI. Indeed, a recent study has defined a functional zone for the RXR motif based on its distance to the membrane (Shikano and Li, 2003). Our results for Ii confirm that the RXR motif is inactive when too close to the membrane (Fig. 7). In the GABAB1 subunit, removal of the RSRR motif from its active zone by the interaction with the B2 subunit was recently proposed as a possible mechanism explaining ER egress of the receptor (Gassmann et al., 2005).

The interplay between the functional regions of Ii and the class-II β cytoplasmic tail remains to be characterized. Although very small, the three-amino-acid cytoplasmic tail of DRβ acquires functional properties, in contrast to the fully truncated version with only the tyrosine residue. The lateral diffusion properties of the two molecules in the membrane could be drastically different and might influence the behaviour of Ii (Capps et al., 2004). However, before one can understand the role of the DRβ chain, the means and chaperones involved in Iip35 retention will need to be identified. Despite phosphorylation of Ser8 and 14-3-3 protein binding, Iip35 accumulates in the ER in the absence of class-II molecules. If one accepts that 14-3-3 proteins bind to Ii at the ER level and thus prevent the interaction with β-COP (O'Kelly et al., 2002) then a second ER-retention mechanism must prevail in the absence of class-II molecules. A COPI-independent mechanism has been proposed to explain the retrograde transport of toxins and glycosyltransferases in the presence of COPI inhibitors (Girod et al., 1999; White et al., 1999). However, we cannot exclude the possibility that, in the absence of class II, the 14-3-3-associated Ii trimer never leaves the ER and is rather retained by dynamic clustering and exclusion from exit sites, as described for Tapasin (Andersson et al., 1999; Pentcheva et al., 2002). Such retention would probably not be mediated by 14-3-3 proteins, because Schwappach and co-workers recently showed that a chimeric CD4 fused to a high-affinity 14-3-3-interacting peptide was not retained in the ER (Yuan et al., 2003). There is no evidence so far for the existence of any COPI-independent mechanism of ER retention in the case of Ii. The role of class-II molecules in preventing such retention is thus impossible to predict. Anyhow, the idea that 14-3-3 proteins shield the RXR motif is difficult to reconcile with the fact that mutation of the phosphorylated residue Ser8 to aspartic acid (Iip35 S8D) prevents 14-3-3 protein binding but allows ER egress if class-II molecules are present (Kuwana et al., 1998).

In conclusion, the mechanism by which the DRβ chain overcomes the ER retention motif of Iip35 remains obscure. Many issues, such as the importance of stoichiometry, the sequence of events in the binding of chaperones, the role of Ii phosphorylation and the exact intracellular trafficking of the p35 isoform, must be tackled before a comprehensive model of class-II maturation can be proposed.

We thank E. O. Long, R. P. Sékaly, O. Bakke, D. L. Hardie, I. C. MacLennan, and P. Cresswell for providing cDNAs and antibodies. H.K. and A.B. were supported by studentships from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR). J.T. is the recipient of a FRSQ scholarship. This work was supported by a Young Investigator Award to J.T. by Boehringer Ingelheim Canada and by a start-up fund from Diabète Québec.

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