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FEBS Letters 586 (2012) 1318–1324

FEBS Letters

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# Expression, purification and assembly of soluble multimeric MHC class II–invariant chain complexes

Dušana Majera [a], Katarina Črnigoj Kristan [a], Jacques Neefjes [c], Dušan Turk [a,b,*], Marko Mihelič [a,b]

[a] Department of Biochemistry, Molecular and Structural Biology and Centre for Protein and Structure Production, Jozef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia
[b] Centre of Excellence CIPKEBIP, Jamova 39, SI-1000 Ljubljana, Slovenia
[c] Division of Cell Biology and Centre for Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

ARTICLE INFO

*Article history:*
Received 7 March 2012
Accepted 14 March 2012
Available online 27 March 2012

Edited by Gianni Cesareni

*Keywords:*
Immune response
MHC class II molecule
Invariant chain
Transmembrane domain
Invariant chain trimerization

ABSTRACT

**Major histocompatibility class (MHC) II molecules are essential for running adaptive immune response. They are produced in the ER and targeted to late endosomes with the help of invariant chain (Ii) trimers. Ii trimerization may be induced by the Ii TM domain. To enable mechanistic and structural studies of MHC class II–Ii assembly, soluble forms of the complexes were expressed. We show that Ii trimerizes in the absence of the transmembrane part, prior to binding of α/β chains. The biochemical analysis supports the suggestion that the MHC class II–Ii complexes are not necessarily trimers of trimers, but that the Ii trimer can also be occupied by one or two MHC class II complexes.**

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## 1. Introduction

The major histocompatibility class (MHC) II–invariant chain (Ii) complexes are transmembrane glycoproteins that play a central role in adaptive immunity [1,2]. They are synthesized in the endoplasmic reticulum (ER) where the nascent MHC class II α and β chains associate with Ii trimers [3,4]. Once fully assembled, the MHC class II–Ii complexes are transported to the late endosomal compartments called MIIC of professional antigen presenting cells (APCs) [5,6]. Here, Ii is degraded in a step-wise manner by the lysosomal cysteine proteases [7,8] resulting in the release of the active MHC class II dimers. Ii consists of several distinct segments that play specific roles in the assembly and cellular localization of MHC class II complexes, including the N-terminal cytosolic tail which contains signals for the delivery of MHC class II into the endocytic route [9], a single transmembrane helix, a trimerisation domain located at the luminal site of the protein and a short segment termed CLIP which associate with the MHC class II peptide binding groove and prevents premature binding of antigenic peptides [10]. Ii exists in two alternatively spliced forms, the p31 and p41 form, which differ by the insertion of a 64 amino acid long domain in the p41 form of Ii [11]. This domain is a strong inhibitor of cysteine endopeptidases suggesting that the invariant chain might regulate its own proteolytic processing [12,13].

The molecular mechanism of MHC class II α (DRA) and β (DRB) chain and Ii association has been extensively studied for the past decades, however the exact mechanism of their assembly still remains unclear. The classic model of the MHC class II–Ii assembly suggests that the Ii first forms a trimer via its trimerisation domain. This trimers then act as a chaperone-like scaffold for the assembly of three α and β chain dimers that concurrently bind to the Ii trimers forming a nonameric complex [14,15]. Recently, Koch et al. challenged this concept by showing that association of Ii with DRA alone precede the binding of DRB and that the Ii trimer can bind only a single MHC class II αβ dimer before leaving the ER for transport into the endocytic route [16–18].

Several studies evaluated the importance of the transmembrane (TM) region in the trimerisation of Ii. It was shown that the TM regions of the Ii strongly self-associate as a trimer [19]. Destabilization of this TM interaction prevented trimerisation of Ii and assembly of the complexes [20]. These studies were performed by mutations of highly conserved amino acid residues within the Ii TM domain and do not fully support the role of the TM domain in the assembly of the complexes.

To unambiguously address the role of the TM domain of the Ii in the assembly of the MHC class II–Ii complexes, we produced soluble complexes lacking the TM region. The analysis of the purified MHC class II–Ii complexes lacking the TM regions showed that soluble Ii still formed a trimer that associate with MHC class II α and β

* Corresponding author at: Department of Biochemistry, Molecular and Structural Biology and Centre for Protein and Structure Production, Jozef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia. Fax: +386 1 477 3984.
*E-mail address:* dusan.turk@ijs.si (D. Turk).

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http://dx.doi.org/10.1016/j.febslet.2012.03.038

chains. These complexes are efficiently secreted by cells allowing rapid purification in quantities sufficient for further biochemical studies.

## 2. Materials and methods

### *2.1. Gene cloning and construction of the expression vectors*

The truncated MHC class II α and β chains (ΔDRA and ΔDRB) containing the N-terminal signal sequence but without the C-terminal TM and cytosolic domains were amplified from the full length DRA and DRB cDNAs, using the following set of primers: 5′CCCA AAGCTTGCC**ACC*ATGG***_CCATAAGTGGAGTCCCT_3′ and 5′CCCAGAATTCT TACTCTGTAGTCT3′ as a forward and reverse primers for ΔDRA and 5′CCCAAAGCTTGCC**ACC*ATGG***_TGTGTCTGAAGCTCCCT_3′ and 5′CCCA GAATTCTTACTTGCTCTGTG CAGATTCAG3′ as a forward and reverse primers for ΔDRB. The underlined sequences correspond to the HindIII (forward primers) and EcoRI (reverse primers) endonuclease recognition site. The region presented in bold is a Kozak consensus sequence. The PCR products were cloned into the pcDNA3 expression plasmid (Invitrogen). The human p31 and p41 forms of Ii (Δp31Ii and Δp41Ii) without the cytoplasmic and TM regions were PCR amplified using 5′CCCAACCGGTCGGCTGGACAAACTGACAGT3′ as a forward and 5′CCCAGG TACCCATGGGGACTGGGCCCAGATC3′ as a reverse primers. The underlined regions represent AgeI and KpnI restrictions sites. The PCR products were cloned into the pHLsec expression plasmid [21] in frame with the N-terminal H2-$\kappa^b$ signal sequence and the C-terminal His-tag.

### *2.2. Expression of truncated MHC class II – invariant chain complexes*

HEK293 cells were maintained in DMEM medium supplemented with 4 mM L-glutamine and 10% fetal bovine serum in a $CO_2$ incubator at 37 °C with 5% $CO_2$.

For the small-scale transfection, the HEK293T and HEK293S GnTl- cells were seeded in a 6-well cell culture plate and transfected with Lipofectamin (Invitrogen) according to the manufacturer's instructions. For large-scale expression the cells were seeded in T150 flasks and cultured in 25 ml medium. When the cells reached 60% confluency, co-transfection using 25 kDa linear PEI (Polysciences Inc.) was performed. Forty micrograms of individual plasmids containing cDNAs for ΔDRA, ΔDRB, or p41 form of ΔIi were mixed in equimolar ratios in a 5 ml of serum-free DMEM. One hundred and twenty micrograms of PEI was added to the mixture. After 10 min of incubation at the room temperature the mixture was added to the cells. After 4 h, the media was exchanged with 25 ml of DMEM without FBS and the cells were cultured for 3 days.

### *2.3. Purification of MHC class II – invariant chain complexes*

The conditioned medium from the HEK293T cells seeded in 20 T150 flasks (approximately 500 ml) was collected and concentrated on the Amicon ultra filtration device and dialyzed against the 30 mM Tris, pH 7.5, 100 mM NaCl, 20 mM imidazol buffer (binding buffer). The sample was applied to the 1 ml HiTrap IMAC FF column (GE Healthcare) charged with $Ni^{2+}$ at a flow rate 1 ml/min. The unbound proteins were washed with the 20 column volumes of the binding buffer. The bound proteins were eluted with the binding buffer containing 300 mM imidazol. The eluted complexes were dialyzed against the 20 mM Tris, pH 7.5 buffer and additionally purified on the Mono Q column (GE Healthcare) equilibrated in the same buffer. The recombinant complexes were eluted with a linear gradient of NaCl from 0 to 0.5 M in the 20 mM Tris buffer, pH 7.5. The eluted samples were desalted by a PD-10 desalting column, concentrated and stored at −70 °C.

### *2.4. SDS–PAGE, Western blotting and N-terminal sequencing*

The purified complexes were analyzed by SDS–PAGE using the 15% Bis–Tris gels and visualized by the Coomassie Brilliant Blue staining. For Western blot analysis, the proteins were electrotransferred to nitrocellulose membrane. The membrane was blocked with 5% (w/v) skim milk in Tris-buffer saline containing 0.05% (v/v) Tween 20 (TBST) for one hour at room temperature. The rabbit polyclonal anti-DRA, anti-DRB [5] and anti-Ii antibodies (a gift from P. Cresswell, Yale University) were added in dilution 1:1000 and incubated for one hour at room temperature. The bound antibodies were detected with the horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and visualized with peroxidase substrate.

For N-terminal sequencing, the proteins separated on SDS–PAGE gel were electrotransfered to the PVDF membrane, and visualized with 0.1% Brilliant Blue G in 1% (v/v) acetic acid and 40% (v/v) methanol. The N-terminal sequencing was performed on Precise Protein Sequencing System 492 (PE Applied Biosystems).

### *2.5. Co-Immunoprecipitation of the complexes*

The IB5 mouse monoclonal antibody against the human DRA or Tu36 mouse monoclonal antibody against DR dimers were added to 1 ml of the conditioned medium and incubated for one hour on the rotary wheel at 4 °C. The antibody–protein complex was isolated by protein A Sepharose beads (GE Healthcare) according to the manufacturer's instructions and analyzed by Western blot using anti-DRA, anti-DRB and anti-Ii rabbit polyclonal antibodies.

### *2.6. Analytical size-exclusion chromatography*

Superdex 200 HR 10/30 (GE Healthcare) size exclusion column was equilibrated in 30 mM HEPES, pH 7.5, 0.3 M NaCl buffer and calibrated with the gel filtration standards (Bio-Rad). The $M_r$ of the complexes was determined from the plots of elution volumes of standards versus log of their $M_r$.

### *2.7. Crosslinking of truncated MHC class II – invariant chain complexes*

Ten micrograms of the Δ(DRA/DRB/p41Ii) complex or the Δp41Ii alone in 20 μl of HEPES, pH 7.5 buffer were mixed with 2.5 μl of 2.3% solution of gluteraldehyde or 5 mg/ml solution of DSS and incubated at the room temperature for 15 min. The crosslinking reaction was terminated by addition of 5 μl of 1 M Tris, pH 7.5. The crosslinked complexes were separated on the 3–8% Tris–Acetate PAGE gels and stained with Coomassie Blue.

### *2.8. Deglycosylation with Endo H*

The samples were treated with endoglycosidase Endo H (New England Biolabs, UK) according to the manufacturer's instructions. The samples were separated by SDS–PAGE and analyzed by Western blotting using anti-DRB antibodies.

## 3. Results and discussion

### *3.1. Design of the expression constructs*

To promote the expression of MHC class II–Ii chain complexes in the soluble form, sequences of the individual chains have been modified. In the case of Ii the N-terminal cytosolic tail and the transmembrane region (encompassing amino acids from G47 to Y72) followed by three consecutive Gln residues were removed.

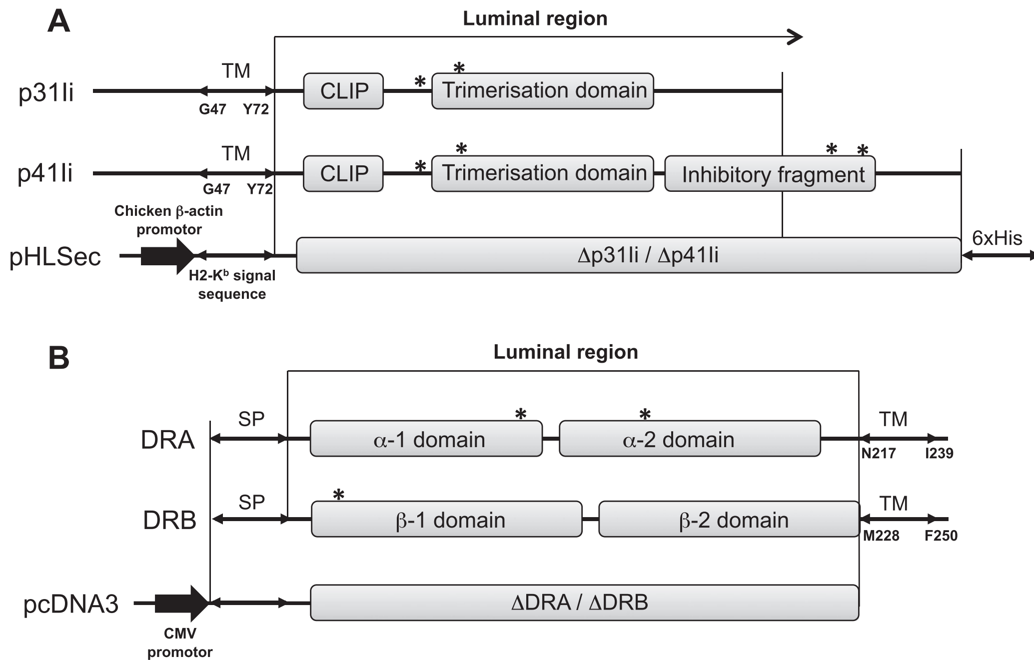


**Fig. 1.** Schematic representation of expression plasmids for (A) MHC class II associated p31 and p41 forms of the invariant chain (Ii) and (B) MHC class II α (DRA) and β (DRB) chains. The regions of DRA, DRB and Ii that were cloned into the expression plasmids are annotated with Δ. SP marks the signal peptide, TM the transmembrane region, ⁎ the sites of N-linked glycosylation and 6xHis the histidine affinity tag.

The remaining luminal part of the Ii starting from G76 was cloned into the pHLSec plasmid in frame with the N-terminal H2-Kb signal sequence (Fig. 1A). The truncated Ii lacks the N-terminal type II signal sequence essential for the delivery of the complexes into the endocytic route and we therefore introduced a new type I signal sequence for insertion in the ER and transport for secretion. In contrast to Ii, the transmembrane regions of MHC class II α and β chains are located at the C-termini. They encompass residues from N217 to I239 and from M228 to F250 of the α (DRA) and β (DRB) chains respectively. To facilitate expression of the soluble DRA and DRB chains both sequences were removed by terminating the chains at amino acid E216 (DRA) and K227 (DRB), respectively. Hence, only the luminal parts of DRA and DRB chains with the native N-terminal signal sequence were cloned into the pcDNA3 plasmid (Fig. 1B). All constructs contain the N-terminal signal sequences that will target the synthesized polypeptide chains into the lumen of ER, where the assembly of the complexes takes place.

### 3.2. Association of soluble MHC class II αβ and Ii in vivo

Individual constructs of the truncated ΔDRA, ΔDRB and ΔIi chains were transfected in HEK293 cells either alone or mixed in different combinations. Expression of individual chains was analyzed by immunoblotting (Fig. 2). When the p41 and p31 isoforms of the ΔIi were transfected alone or in combination with ΔDRA and ΔDRB chains the majority of the invariant chain was secreted (Fig. 2, Anti Ii blots). The absence of the cytosolic targeting signals thus resulted in targeting of the Ii into the secretory pathway [22]. In contrast to the ΔIi, a different distribution pattern was observed when expression of ΔDRA and ΔDRB chains was analyzed. When ΔDRA chain was transfected alone or in combination of only one of the other subunits (ΔDRB chain or the ΔIi), it was only detected inside the cells, whereas expression of all three subunits (the ΔDRA, ΔDRB and ΔIi chains) resulted in secretion as detected by anti-DRA antibodies (Fig. 2, Anti DR blot). Similar results were obtained for ΔDRB. The secreted DRB was detected only when ΔDRB was co-transfected together with ΔDRA and the ΔIi (Fig. 2, Anti DRB blot). When transfected alone or only with the ΔIi or ΔDRA, the ΔDRB chain was retained and detected only intracellularly. Due to the weak affinity and high cross reactivity of DRB antibodies, the intracellular expression of ΔDRB could be detected only by increasing the exposure time for blot development which resulted in the increased background and non-specific staining of other proteins. However, from Fig. 2, (Anti DRB⁎ blot) it can be clearly seen that ΔDRB, when expressed alone or in combination with ΔDRA or ΔIi, is retained intracellularly. We have also observed increased intracellular expression of ΔDRA as well as ΔDRB when transfected with ΔIi alone (Fig. 2, blot Anti DRA and blot Anti DRB), suggesting that ΔIi somehow stabilize individual DRA and DRB. Comparison of ΔDRA, ΔDRB chains and the ΔIi inside the cells and secreted forms shows obvious differences in molecular weight. Since all three chains contain a number of *N*-glycans (Table 1), the deviation in the molecular weight marks conversion of high-mannose to complex *N*-glycans that is the result of transport through the secretory pathway. This also indicates that ΔDRA and ΔDRB when transfected alone or only in pairs are retained in the ER. To provide further support that the differences in the molecular weight arise from the localization and N-glycosylation pattern, we have analyzed ΔDRB transfected into the HEK293S GnTI- cell line which is deficient in *N*-acetylglucosaminyltransferase I activity and therefore lacks complex type *N*-glycans. When ΔDRB was cotransfected with ΔDRA and the ΔIi in HEK293S GnTI- cells, the apparent molecular weight of the secreted ΔDRB is identical to the molecular weight of the intracellularly accumulated ΔDRB (Fig. 3A). The weight of intracellular ΔDRB was further decreased by the Endo H treatment, which removes the basic mannose glycan attached to the proteins in the ER during early stages of their synthesis, whereas secreted ΔDRB (when cotransfected with ΔDRA and ΔIi) is Endo H resistant. The ER localization of ΔDRB, when transfected alone, was additionally confirmed by staining fixed cells with anti-DRB antibodies before analyses by confocal microscopy (Fig. 3B). Also ΔDRA was retained in the ER according to biochemical and microscopy criteria. These observations are consistent with the previous reports that MHC class II dimers can

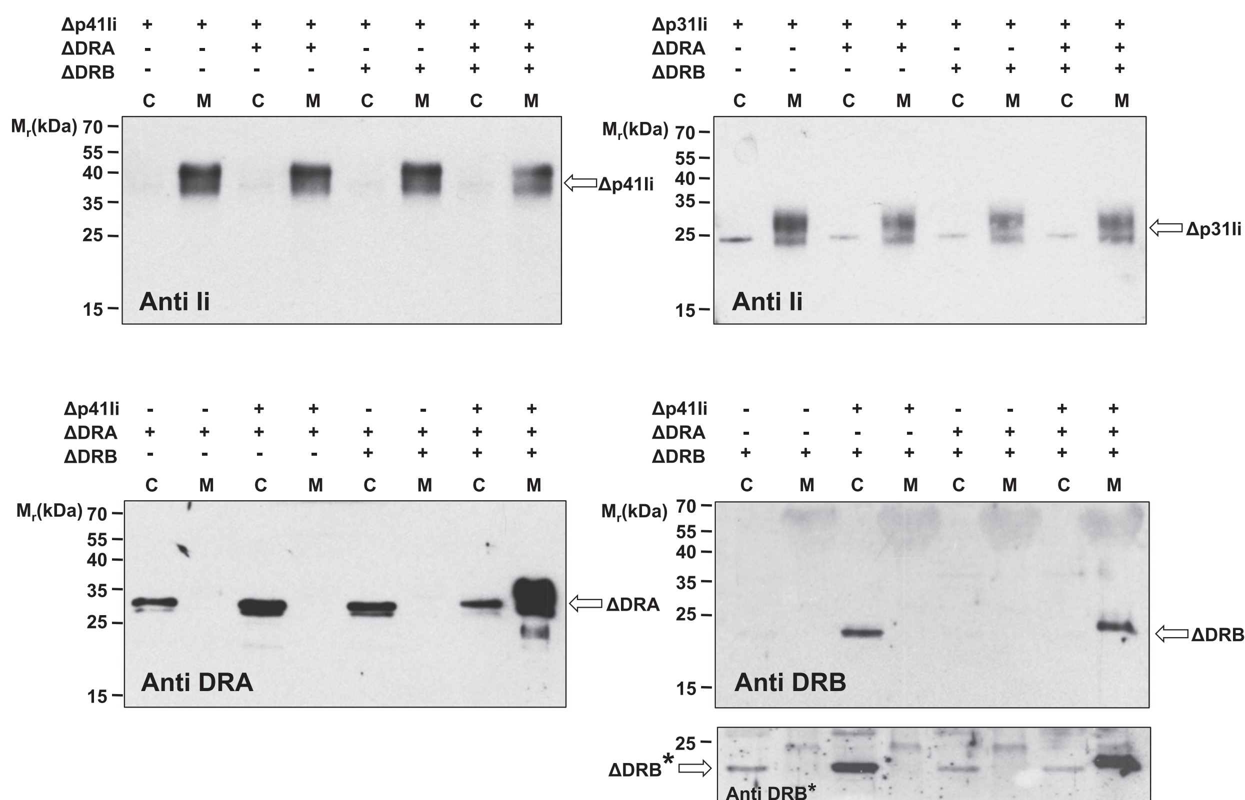


**Fig. 2.** Expression analysis of soluble MHC class II and Ii proteins. Western blot analysis of intra- (C) and extracellular (M) expression of Δp41Ii, Δp31Ii, ΔDRA and ΔDRB after transfection of HEK293T cells with different combinations of plasmids. The proteins were detected using the anti-Ii, anti-DRA and anti-DRB antibodies. Plus (+) and minus (−) signs indicate which combination of plasmids was used in the transfection experiments. The anti-DRB blot developed with extended exposure time is marked with ⁎.

**Table 1**
Calculated molecular weights and number of potential N-glycosylation sites of truncated forms of MHC class II DRA and DRB and p41 invariant chain.

| | Mr (kDa) | N-glycosylation sites |
|---|---|---|
| ΔDRA | 22 | 2 |
| ΔDRB | 23 | 1 |
| Δp41Ii | 25 | 4 |
| $\Delta(p41Ii)_3$ | 75 | 12 |
| $\Delta(p41Ii)_3\Delta(DRADRB)$ | 120 | 15 |
| $\Delta(p41Ii)_3\Delta(DRADRB)_2$ | 165 | 18 |
| $\Delta(p41Ii)_3\Delta(DRADRB)_3$ | 210 | 21 |

fold and leave – though inefficiently – the ER whereas the free DRA and DRB chains are retained in the ER [15,23].

The existence of the complex between secreted ΔDRA, ΔDRB and the ΔIi was verified by immunoprecipitation using Tu36 mouse monoclonal antibody that recognizes only properly folded and loaded MHC class II DR dimers and the 1B5 mouse monoclonal antibody recognizing DRA [5]. Immunoblots of immuno-isolated fractions using anti-DRA, DRB and Ii antibodies showed that all three chains associated in a complex (Fig. 4).

### *3.3. Large-scale expression and purification of soluble MHC class II – Ii invariant chain complexes*

The MHC class II–Ii complexes secreted from HEK293T and glycosylation defective HEK293S GnTl- cell lines were purified on the Ni-chelating column. We have attached a His-tag at the C-terminus of Ii for isolation purposes. Co-purified ΔDRA and ΔDRB should then be in a complex with ΔIi. To separate the soluble MHC class II–Ii chain complexes from the soluble Ii alone that might co-purify on the Ni-affinity column, the samples were subjected to further purification. The calculated isoelectric points for the truncated MHC class II–Ii complex and the Ii are 5.71 and 8.05, respectively which suggest that they can be separated by ion-exchange chromatography (Mono-Q) performed at pH 7.5. At this pH, the complex is negatively charged and should bind to the positively charged ion-exchange carrier, whereas the more acidic empty Ii trimer should theoretically flow through. The fractions corresponding to the major eluted peak from the Mono-Q column were collected and analyzed by SDS–PAGE (Fig. 5A). The positions of ΔDRA, ΔDRB and ΔIi on the PAGE-gel were identified by combining the results of the Western blot analysis and the N-terminal amino acid sequencing. The N-terminal sequences confirmed the presence of all three chains in the sample (Fig. 5B).

The molecular weights of ΔDRB – as deduced from SDS–PAGE analysis – corresponded to the theoretical masses (including the carbohydrates) calculated from the amino acid sequences (Table 1). The estimated molecular weight for ΔDRA is higher than calculated, however expression in HEK293S GnTl- cells results in the formation of a sharp band at 30 kDa (estimated Mw). The apparent weight of the glycosylated p41 form of ΔIi is between 35 and 40 kDa, which is substantially higher than the theoretical value. Expression of the complexes in N-glycosylation deficient cell line resulted in the decrease of the molecular weight of the Ii, however, the Ii is still present in several bands with higher weight than expected. The single O-glycosylation site in the Ii that contributes a considerable number of negative charges may cause the faster migration by SDS–PAGE. Immunoblot analysis of the purified complexes using anti-His antibodies, which recognize the affinity tag attached to the C-terminus of the Ii, showed identical staining as Coomassie Blue. By the N-terminal sequencing, we were able to identify only a single amino acid sequence that starts at the last three amino acids of the H2-$\kappa^b$ signal sequence followed by the

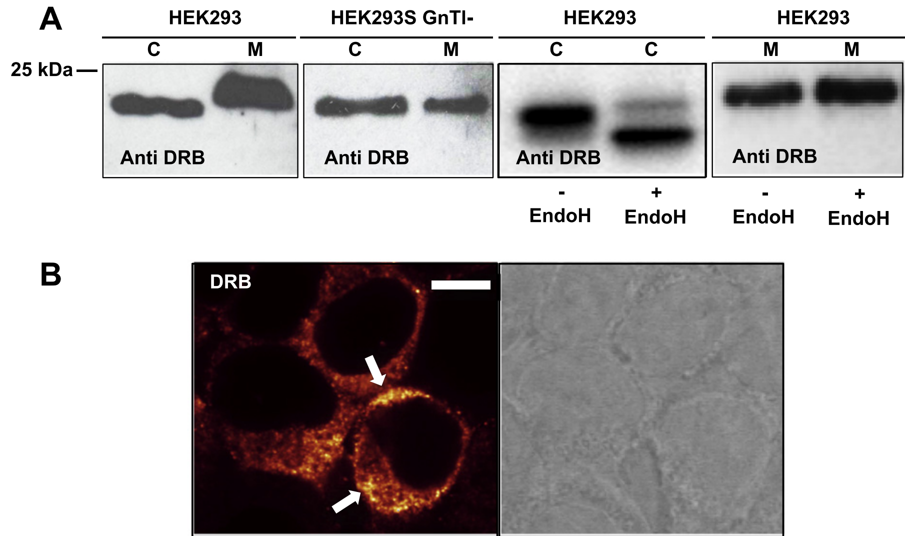


**Fig. 3.** Analysis of the molecular size, cellular localization and glycosylation pattern of ΔDRB. (A) ΔDRB was transfected together with ΔDRA and the ΔIi in HEK293T and HEK293S GnTI- cells and analyzed by anti-DRB immunobloting. C and M annotates intracellular and extracellular expression. Intracellular and secreted ΔDRB was additionally treated with Endo H. (B) Immunofluorescence staining of ΔDRB transfected alone in HEK293T cells and imaged by confocal microscopy. The arrow indicates the accumulation of the ΔDRB inside the ER. Bar: 100 μm.

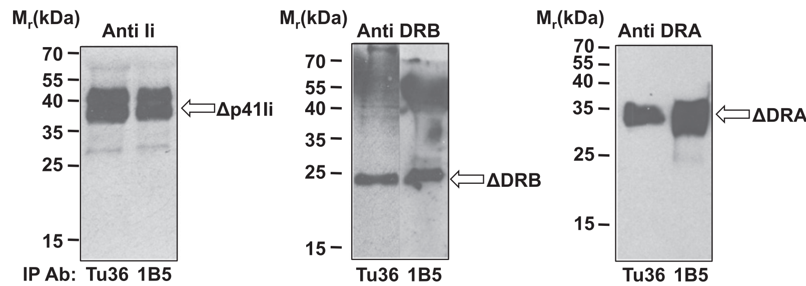


**Fig. 4.** Secreted MHC class II molecules lacking the transmembrane region associate with the soluble Ii. Western blot analysis of immunoprecipitated soluble MHC class II–Ii complexes is shown. The complexes were immunoprecipitated with Tu36 and 1B5 mouse monoclonal antibodies. The individual chains of the complex were detected with the rabbit polyclonal antibodies specific to individual chain.

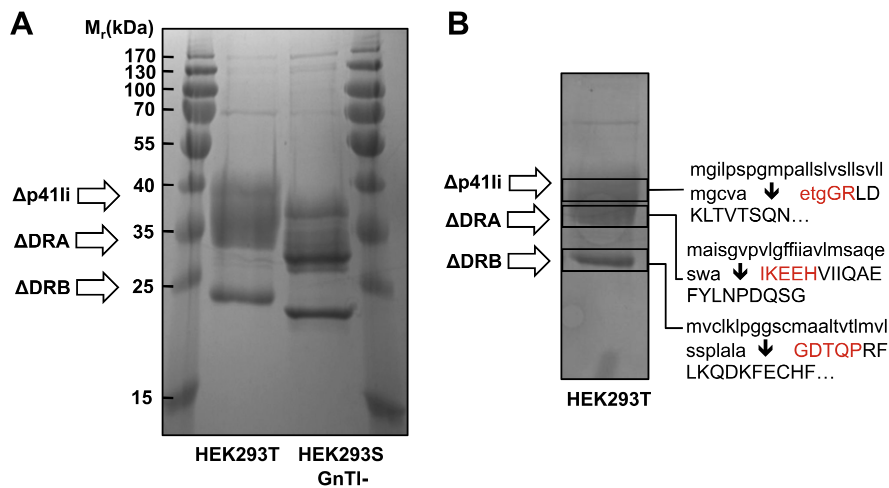


**Fig. 5.** SDS–PAGE analysis and N-terminal amino acid sequencing of MHC class II–Ii chain complexes. (A) The purity of the complexes expressed in HEK293T (lane 1) and HEK293S GnTI- cell line (lane 2) was analyzed by SDS–PAGE. The positions of ΔDRA, ΔDRB and the Δp41Ii are marked. (B) The complex purified from cotransfected HEK293T cells was transferred to PVDF membrane. The bands that were cut out and sequenced are shown in frames. The determined N-terminal amino acid sequences are shown in red. The predicted cleavage sites for signal peptidase are marked with arrows.

Ii sequence. This further indicates that multiple and diffused bands corresponding to the p41 Ii form of the invariant chain are the consequence of posttranslational modifications and not of proteolytic degradation. The N-terminal sequence was also determined for

ΔDRA and ΔDRB. In both cases the sequence matched the sequences of DRA and DRB lacking the signal peptide (Fig. 5b).

*3.4. Oligomeric state of soluble MHC class II–Ii complexes*

To analyze the stoichiometry of the expressed proteins, we assessed the molecular weight of purified proteins by analytical size exclusion chromatography and crosslinking experiments. The purified proteins eluted from the Superdex 200 HR size exclusion column in a single symmetric peak with elution volume around 10 ml (Fig. 6A). The apparent molecular weight of these complexes was determined from the calibration curve obtained by plotting the elution volumes of the protein standards versus logarithm of their molecular weight (Fig. 6B). The elution volumes of the ΔDRA-DRBp41Ii complexes purified from HEK293T and HEK293S GnTl- cell lines correspond to the apparent molecular weights of 290 and 273 kDa. Since only a single peak was eluted from the size-exclusion column these results suggest that the expressed ΔDRA, ΔDRB and the ΔIi associate in high-molecular weight complex. (It should be noted that the estimation of molecular weight of proteins by size exclusion chromatography is reliable only for globular proteins as it depends on the protein radius and its hydrodynamic properties and should therefore be considered only as an approximation of the actual weight.)

To independently confirm the oligomeric structure of the soluble MHC class II–p41Ii complex, the purified complex was cross-linked with gluteraldehyde and DSS. Both crosslinkers gave the same results so only data with gluteraldehyde are shown (Fig. 7). To reduce the unspecific crosslinking between complexes, the concentration of the protein used in these experiments was reduced below 0.5 mg/ml. The molecular weight of the cross-linked soluble p41Ii is 100 and 75 kDa for glycosylated and non-glycosylated forms of the protein. This weight is in agreement with the expected molecular weight of the Ii trimers. Since the trimeric structure was formed in the absence of the transmembrane and cytosolic parts of the Ii, it is evident that the transmembrane region and cytosolic tail of Ii are not critical for the formation of the nonameric complex. The luminal segment of Ii should also contain information that induces trimerization. The crosslinking of the Δ(DRADRBp41Ii) complexes showed two major bands of molecular mass of 130 and 180 kDa for glycosylated form of the complexes, and two bands of 110–140 kDa for underglycosylated forms of the complexes. However, a minor bands of 210 and 170 kDa were also observed. A protein complex of 209 kDa would correspond to the calculated molecular weight of the expected $(DRA–DRB–Ii)_3$ nonameric form. The results from the cross-linking experiments suggest considerable heterogeneity in the binding of HLA–DRAB complexes on the Ii trimer with a preference for one and two MHC class II complexes corresponding to molecular weights of 120 and 160 kDa, respectively (Table 1).

With two independent experiment approaches, namely size-exclusion chromatography and crosslinking, we have estimated the composition of secreted MHC class II–Ii protein complexes. The size exclusion experiments suggest that the complex resembles a trimer of trimers with three MHC class II complexes associated to three Ii chains that form the core of the complex. Chemical cross-linking and more precise molecular weight determination by SDS–PAGE reveals a more complex picture with an Ii trimer associated with mainly one or two MHC class II complexes and only a small proportion in the nonameric state. This would correspond to the recent observations Koch and coworkers [18], who suggested that the stoichiometry of the MHC class II–Ii chain composition may not be a trimer of trimers (a DRA–DRB–Ii trimer) [3] but a pentamer composed of a trimer of Ii with a one pair of Δ (DRADRB) bound. As Ii is present in the ER in considerably higher amounts than DRA and DRB chains, two options exist. MHC class II-Ii complexes are only considered correctly folded to leave the ER when in a nonameric complex (the trimer of trimers) or the Ii trimer can leave the ER when already one or two MHC class II complexes are associated. Our results suggest that the latter option is most likely. The ratio between fully and partially MHC class II–Ii loaded complexes may depend on the relative amounts of Ii and MHC class II DRA and DRB expressed, which may vary between cells and likely depending on the DRA and DRB sequences. The existence of intermediate MHC class II–Ii complexes of various stoichiometries was also observed before. Jasanoff et al. showed that under in vitro conditions MHC class II dimers bind to the Ii trimmers independently and that nonameric complexes are formed only in the excess of MHC class II dimmers [24].

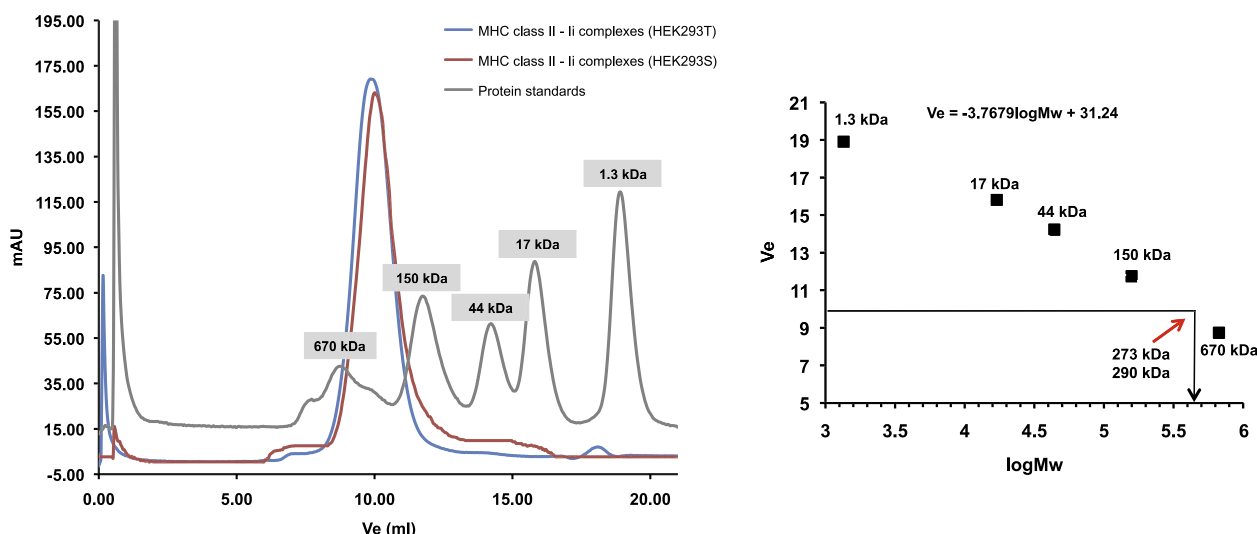


**Fig. 6.** Assessment of molecular weight of MHC class II–41Ii complexes using size exclusion chromatography. (A) Superdex 200 HR elution profiles of MHC class II–p41Ii complexes expressed in HEK293T (blue) and HEK293S GnTl- cells (red). The elution profile of protein standards (thyroglobulin 667 kDa, gamma-globulin 158 kDa, ovalbumin 44 kDa, myoglobin 17 kDa and vitamin B12 1.35 kDa) is shown in gray. (B) Calibration plot obtained from plotting the elution volume of standards versus their molecular weight. The elution volume and calculated molecular weight for MHC class II–p41Ii complexes are marked.

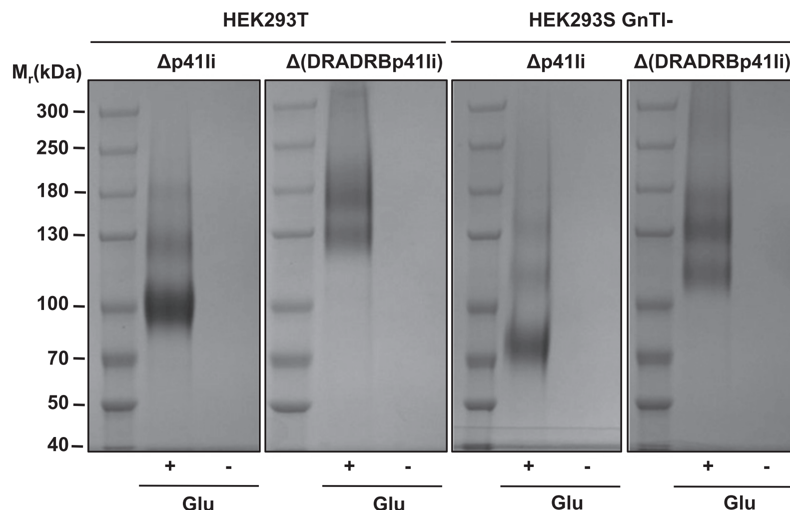


**Fig. 7.** Crosslinking of the soluble MHC class–p41Ii and p41Ii complexes. The lanes with the gluteraldehyde (Glu) crosslinked material are marked on the top with "+" and the lanes without crosslinking with "–". The proteins were separated on 3–8% Tris–Acetate gels and stained with Coomassie Blue.

## 4. Conclusions

Several studies that were addressing the role of Ii TM region in the trimerisation of Ii and assembly of MHC class II–Ii complexes suggested that this domain plays an essential role in these processes [19]. These studies utilized point mutations in the highly conserved patch of polar amino acids in the TM domain of the Ii. These mutations possibly disrupted the interaction between the transmembrane helices of Ii, which might disturb the orientation of Ii in the luminal domain to prevent trimerisation and binding of MHC class II dimers. Here we analyzed the assembly and oligomeric state of the Ii and MHC class II complexes lacking the transmembrane region. We have shown by in vivo and in vitro analysis that the Ii chain lacking the transmembrane and cytoplasmic regions is efficiently secreted as a trimer. Moreover, we have shown that soluble Ii can assemble and be secreted with soluble DRA and DRB in complexes that may be more heterogeneous than assumed previously.

## Acknowledgements

This work was financially supported by the Marie Curie Fellowship MRTN-CT-512385 (to D.M.) and Slovenian Research Agency.

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