Interaction of MHC class II molecules with the invariant chain: role of the invariant chain (81–90) region

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Association of the invariant chain (Ii) with MHC class II α and β chains is central for their functionality and involves the Ii CLIP(81–104) region. Ii mutants with an antigenic peptide sequence in place of the CLIP region are shown to form $\alpha\beta$ Ii complexes resistant to dissociation by SDS at 25°C. This reflects class II peptide binding site occupancy, since substitution of the major anchor residue within the antigenic peptide sequence of one of these Ii mutants abolishes its capacity to form SDS-stable heterotrimers. Therefore, CLIP location within Ii is compatible with CLIP access to the class II binding groove. However, in wild-type Ii this access does not lead to a tight association, which seems to be affected by the Ii 81–90 region. This region, together with a region C-terminal of CLIP, is shown to contribute to Ii association with HLA-DR1 molecules. Thus, Ii mutants with non-HLA-DR1 binding sequences in place of the CLIP(87–102) region can still associate with HLA-DR1 molecules and inhibit peptide binding.

Keywords: CLIP/Ii chain association/inhibition of peptide binding/*in vitro* translation/MHC class II

Introduction

Classical MHC class II molecules are synthesized in the endoplasmic reticulum (ER), where their α and β subunits associate with trimers of the invariant chain (Ii) to form nonameric ($\alpha\beta$ Ii)₃ oligomers (Roche *et al.*, 1991; Lamb and Cresswell, 1992). At this stage Ii inhibits premature binding to class II molecules of endogenous peptides (Roche and Cresswell, 1990; Teyton *et al.*, 1990) or of unfolded polypeptides (Busch *et al.*, 1996), promotes correct folding of the oligomer and its egress from the ER (Layet and Germain, 1991; Anderson and Miller, 1992). Following transfer to the Golgi and *trans* Golgi network, the ($\alpha\beta$ Ii)₃ complex is sorted for the endocytic pathway due to signals essentially carried by the Ii cytoplasmic tail (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Pieters *et al.*, 1993; Odorizzi *et al.*, 1994). Here, the Ii chain is degraded, probably in a sequential manner. The shortest fragment of Ii found associated with class II molecules is CLIP (class II associated invariant chain peptides) (Rudensky *et al.*, 1991; Chicz *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992). Class II–CLIP complexes represent a natural intermediate in class II maturation (Morkowski *et al.*, 1995). Exchange of CLIP

for antigenic peptides is largely dependent on HLA-DM molecules located in endocytic compartments (Roche, 1995; Kropshofer *et al.*, 1997). After peptide loading, class II molecules reach the cell surface, where they present bound peptides to CD4⁺ T cells (Unanue, 1992).

Ii is a type II transmembrane protein (Claesson *et al.*, 1983). Four different forms of Ii are found in humans, of which the shortest and most abundant is p33, which carries all the functions described above (Wolf and Ploegh, 1995). The capacity of Ii to form homotrimers seems to be required for an efficient endosomal localization of $\alpha\beta$ Ii complexes (Arneson and Miller, 1995; Germain *et al.*, 1996) and probably as a consequence, for the presentation of some antigens by class II molecules (Bertolino *et al.*, 1995). Trimerization is thought to involve the C-terminal two-thirds of the luminal part of Ii (Bijlmakers *et al.*, 1994b; Bertolino *et al.*, 1995; Park *et al.*, 1995; Gedde-Dahl *et al.*, 1997), which are protease-resistant and form an α -helical domain (Park *et al.*, 1995). The transmembrane domain may also contribute to the oligomerization process, as naturally occurring class II complexes with a N-terminal fragment of Ii appear to retain the nonameric structure (Amigorena *et al.*, 1995; Newcomb *et al.*, 1996). The N-terminal third of the luminal part of Ii is susceptible to proteases and by itself does not seem to constitute a structured domain (Park *et al.*, 1995). This domain appears to be flexible enough to allow access of the CLIP region, which is part of it, to the class II binding groove (Park *et al.*, 1995; Cresswell, 1996).

Recent X-ray analysis of an HLA-DR3–CLIP crystal has demonstrated that residues (89–101)—hereafter referred to as ‘core CLIP’—can bind essentially as a conventional antigenic peptide in the groove of class II molecules, whereby Met91 and Met99 represent the two primary anchor residues (Ghosh *et al.*, 1995). Class II molecules of this complex were found to have the same structural organization as HLA-DR1 complexed with HA(306–318) peptide (Stern *et al.*, 1994; Ghosh *et al.*, 1995). CLIP has been functionally dissected into two regions: the N-terminal portion, amino acids (aa) 81–98, is able to compete with the superantigen *Staphylococcus aureus* enterotoxin B for class II binding, while the C-terminal region (92–105) inhibits antigenic peptide binding to HLA-DR molecules (Kropshofer *et al.*, 1995a). Interestingly, CLIP(81–105) has a higher off rate than CLIP(90–105), suggesting that the N-terminal portion of CLIP reduces the stability of its interaction with class II molecules and thereby increases the availability of class II molecules for antigenic peptides (Kropshofer *et al.*, 1995a,b), a finding which is in agreement with other studies (Urban *et al.*, 1994; Adams and Humphreys, 1995). HLA-DM molecules might either increase this instability or the efficiency of the loading process of antigenic peptides (Kropshofer *et al.*, 1997).

The CLIP region (81–104) in the intact Ii also seems to play important roles. Residues 96–104 of human Ii were identified as essential for association with class II molecules by the means of Ii truncations analysed by *in vitro* translation (Bijlmakers *et al.*, 1994b). Studies using deletions and truncations of mouse Ii analysed in transfected COS cells (Freisewinkel *et al.*, 1993; Romagnoli and Germain, 1994), mapped this interaction to 82–107 and 87–110, respectively. The same region of Ii was also found to be required for inhibition of peptide binding to class II molecules (Bijlmakers *et al.*, 1994b; Romagnoli and Germain, 1994) and to be necessary for egress of the $\alpha\beta$ Ii complexes from the ER (Romagnoli and Germain, 1994). Using synthetic peptides and recombinant or proteolytic fragments of Ii, recent studies have demonstrated that other parts of the Ii chain also interact with class II heterodimers, albeit with a lower affinity than CLIP (Park *et al.*, 1995; Vogt *et al.*, 1995; Newcomb *et al.*, 1996).

It was recently shown that CLIP on its own can promote class II folding and egress from the ER as previously demonstrated for Ii (Zhong *et al.*, 1996). This does not necessarily imply that the CLIP region of Ii associates in the same way as CLIP with class II molecules in the ER. Although the binding of CLIP peptides to various MHC class II allele products has been extensively studied, it is still unknown whether the CLIP region in intact Ii has access to the binding groove of class II molecules (Cresswell, 1996). It has been suggested that the CLIP region could inhibit peptide binding either by occupying the peptide-binding groove of associated class II molecules and/or by promoting a conformation in which class II molecules would be unable to bind peptides. To address these questions and to analyse in detail the contribution of the CLIP region of Ii with regard to class II association and peptide-binding inhibition, we have designed a series of Ii mutants. Overlapping parts of the CLIP region have been replaced in these mutants either by the sequence of the HA(306–318) peptide known to bind DR1 molecules or by sequences of different peptides lacking this ability. We used *in vitro* translation of these mutants together with DR1 α and β chains in the presence of microsomes to study association between these proteins and peptide-binding inhibition in the ER. Our results show that the location of CLIP within Ii is compatible with its access to the peptide-binding site of class II molecules, but the tight association between the core CLIP segment and the class II groove appears however to be hindered by Ii 81–90 region. Two regions of Ii, located on each side of the core CLIP region (the 81–90 segment and an area located in the C-terminal two-thirds of Ii luminal domain), are shown to contribute to the association with DR1 molecules. Finally, provided that the length of Ii is maintained, the CLIP(87–102) sequence (which encompasses the core CLIP) is not required for Ii association with class II molecules and Ii inhibition of peptide binding.

Results

Characterization of mutated forms of Ii translated *in vitro*

Mutants of Ii were generated in which the sequence encoding the CLIP region was deleted to different extents

Fig. 1. Human p33 Ii and the Ii mutants used in this study. **(A)** Schematic representation of wild-type Ii and of the mutants constructed. The p33 form of human wild-type Ii is represented showing the transmembrane region (TM), the two N-linked carbohydrate chains (positions 114 and 120), and the sequence of the CLIP region in which the core CLIP sequence is underlined (residues 89 to 101). A set of deletions in the CLIP region were made as described in Materials and methods, creating a new *Kpn*I site in place of the deleted sequences. Different oligonucleotides were introduced in the *Kpn*I site (its position is underlined and it encodes GT). They encode the inserted sequences presented. Another set of mutants was generated using the *Hind*III site (its position is underlined and it encodes KL), which is part of the cDNA encoding the wild-type Ii, to generate the Δ (82–89) mutants and to insert IR4 or IR5 sequences. **(B)** Endoglycosidase H digestion of Ii mutants translated *in vitro*. mRNAs encoding the nine indicated Ii mutants were translated *in vitro* in the presence of microsomes. After solubilization of the microsomes, translation products were subjected to immunoprecipitation with PIN1 mAb (anti Ii-cytoplasmic tail). Polypeptides recovered with PIN1 were either mock-digested (–) or digested with endo H (+) to remove N-linked carbohydrate chains. Numbers of N-linked carbohydrate chains carried by each product are indicated for the Δ (81–102) mutant. The position of dimers of mutants is shown. Migration and molecular weights (MW) in kDa of protein markers are indicated on the right.

(residues 81–102, 87–102 or 91–102) and replaced by sequences encoding either other peptides, termed IR, or the HA(306–318) peptide (Rothbard *et al.*, 1988), termed HA (Figure 1A). cDNAs encoding these constructs were used for production of mRNA *in vitro*. Translations were performed in rabbit reticulocyte lysates in the presence of microsomes as ER equivalents. After translation, microsomes were recovered by centrifugation and solubilized in detergent. Translation products were immunoprecipitated with PIN1, a mAb which is specific for the cytoplasmic tail of Ii, and analysed by SDS-PAGE.

After immunoprecipitation with PIN1 mAb, three bands were observed for each Ii mutant (Figure 1B). The use of BU45 mAb (which is specific for the C-terminal part of Ii) instead of PIN1, generated similar patterns (data not shown). Treatment with Endo H revealed that the most abundant band with the slowest mobility corresponded to

Table I. Comparison of different Ii mutants for their ability to associate with HLA-DR1 molecules^a

	Length (aa)	Ii contact region ^b			HLA-DR1 association
		(81–90)	(90–102)	C-ter ^c	
p33Ii	216	+	+	+	++++
Δ(81–102)	196	–	–	(–)	–
Δ(81–102)IR1	211	–	–	(–)	–
Δ(81–102)IR1bis	216	–	–	+	+
Δ(87–102)	202	+/-	–	(–)	+
Δ(87–102)IR1, 2 or 3	217	+/-	–	(+)	++
Δ(91–102)	206	+	–	(–)	++
Δ(91–102)IR1, 2 or 3	221	+	–	(+)	++++
Δ(82–89)	208	–	+	(+)	+++
Δ(82–89)IR4 or 5	216	–	+	+	+++
Δ(81–102)HA	211	–	HA	(–)	++++
Δ(87–102)HA	217	+/-	HA	(+)	++++
Δ(91–102)HA	221	+	HA	(+)	++++

^aEfficiency of HLA-DR1 association with the different Ii mutants was estimated from quantitation of SDS–PAGE gels from at least three independent experiments for each mutant. Capacity of Ii mutants for association with DR1 molecules was expressed as a percentage of wild-type capacity: –, <10%; +, 10–30%; ++, 30–50%; +++, 50–70%; +++++, >70%.

^bPresence (+); partial presence (+/-); absence (–); or replacement by the HA(306–318) peptide sequence (HA) of the indicated Ii region is given for each Ii mutant (see Figure 1A).

^cC-ter stands for the C-terminal region of CLIP. (–) indicates that the C-terminal region of CLIP is present within a particular Ii mutant but might be misplaced due to a deletion N-terminal to this region. (+) indicates that despite a lack of eight residues or addition of either one or five residues as compared with wild-type Ii, the C-terminal contact region of the mutant in question might be at the same spatial location as in wild-type Ii (see text for details).

the full-length product carrying, as expected, two N-linked carbohydrate chains (Figure 1B, band 2). The two lower bands were much less abundant and carried either one (Figure 1B, band 1) or no carbohydrate chain (band 0). For every mutant, the shift observed for each N-linked carbohydrate chain added was 4 kDa, as expected. Homodimers of each Ii mutant were observed (Figure 1B) as previously reported for the wild-type Ii chain (Bijlmakers *et al.*, 1994a) and C-terminal truncations of Ii (Bijlmakers *et al.*, 1994b). Dimerization could result from engagement of Cys28 in disulfide bridges. However, formation of such bridges may be dispensable for dimerization as an N-terminal truncation of Ii lacking this cysteine has been recently reported to have kept the ability to form homodimers (Gedde-Dahl *et al.*, 1997). All the other mutants of Ii used in the present study were also analysed in this way and behaved similarly (data not shown). Their apparent molecular weight corresponded to their predicted one (length in aa is given in Table I). These mutants thus appeared to be correctly translated, glycosylated in the microsomes and—based on antibody recognition—correctly folded.

Replacement of the CLIP region of Ii by a class II binding peptide sequence leads to the formation of SDS-resistant αβIi complexes

There has been no direct demonstration that the CLIP region of Ii has access to the class II binding groove. To address this question, we designed Ii mutants in which the CLIP region was substituted by the HA(306–318) peptide sequence (HA) from influenza virus haemagglutinin, which has a good affinity for DR1 molecules (Rothbard *et al.*, 1988; Sette *et al.*, 1995). Considering that the 81–89 part of the CLIP peptide can reduce the stability of class II–peptide complexes (Kropshofer *et al.*, 1995b) and that Met91 represents the first of the two primary anchor residues of the core CLIP(89–101)

sequence, we focused on the three following regions: 81–102, representing almost the entire CLIP region; 91–102, representing essentially the core CLIP region; and 87–102, as an intermediate situation. Mutants were generated in which each of these three regions was replaced by the HA sequence (see Figure 1A). Binding of HA peptide to DR1 heterodimers produced *in vitro* makes them resistant to dissociation by 4% SDS at 25°C (Bijlmakers *et al.*, 1994a; Hedley *et al.*, 1994; see also Figure 3). We reasoned that if the location of CLIP within Ii allows its access to the peptide binding site of class II molecules, mutants with the HA sequence should form SDS-resistant heterotrimers with DR1 α and β chains.

The three Ii mutants, Δ(81–102)HA, Δ(87–102)HA and Δ(91–102)HA, were found to associate very efficiently with DR1 molecules produced *in vitro* (Figure 2). SDS-resistant complexes were observed with the three mutants when material was eluted at 25°C from TU36, 1B5 and PIN1 mAbs (Figure 2A and B, see arrow). They were less apparent for the Δ(81–102)HA mutant eluted from PIN1 (Figure 2B), but were seen on longer exposures of the gels (not shown). The amount of SDS-resistant complexes was consistently found to be as follows: Δ(87–102)HA > Δ(91–102)HA > Δ(81–102)HA. These complexes dissociated after heating to 95°C (Figure 2A and B, compare boiled with non-boiled lanes). To determine their composition directly, the SDS-resistant bands were cut out from gels, boiled in SDS-sample buffer and run on a second SDS–PAGE (Figure 2D). The results obtained show that these complexes were composed of DR1 α and β chains associated with the respective Ii mutant. The stoichiometry of these associations was estimated by phosphorimaging. We found a 1:1:1 ratio in the case of Δ(87–102)HA and of Δ(91–102)HA. The low abundance of the complexes obtained with Δ(81–102)HA prevented estimation which is nevertheless likely to be the same. These conclusions are in full agreement with the apparent

Fig. 2. The CLIP region of Ii has access to the peptide binding site of HLA-DR1 molecules. (A and B) Wild-type Ii and the three indicated mutants of Ii containing the HA sequence were translated *in vitro* together with DR1 α and β chains. Detergent-lysed microsomes of these translations were divided into three parts and immunoprecipitated with mAbs 1B5 (anti-DR α), TU36 (anti-DR $\alpha\beta$), and PIN1 (anti-Ii) (one part each). One-half of the protein A- or G-bound material was eluted at 95°C (B), the other half at 25°C (NB). The amount of lysed microsomes used per lane was thus identical. Arrows point to SDS-resistant complexes, which disappear when material was eluted at 95°C (B). (C) Wild-type Ii and the mutants $\Delta(87-102)$ HA Y $\rightarrow$ D and $\Delta(87-102)$ HA were individually translated *in vitro* together with DR1 α and β chains. Detergent-lysed microsomes of the translations were divided into two equal parts and immunoprecipitated with mAbs TU36 and PIN1. Half of the protein A- or G-bound material was eluted at 95°C (B), the other half at 25°C (NB). (D) Determination of the composition of the SDS-resistant complexes. SDS-resistant complexes obtained with the three mutants [$\Delta(81-102)$ HA, $\Delta(87-102)$ HA and $\Delta(91-102)$ HA] on a gel similar to the one presented in (A) were cut out from the gel, boiled in SDS-sample buffer and loaded on a second SDS-PAGE.

molecular weight of these complexes (~95–98 kDa) and with their recognition by the three different mAbs used. Pulse-chase analysis of COS cells transiently expressing $\Delta(87-102)$ HA together with DR1 α and β chains gave rise to similar association and formation of SDS-resistant complexes (data not shown). In addition, we observed that the heterotrimers acquired Endo H-resistant glycans with time, indicating that they were efficiently transported from the ER and thus that $\Delta(87-102)$ HA was correctly folded (data not shown).

The SDS-resistance of these heterotrimers can be interpreted as the result of direct interaction of the HA sequence with the peptide-binding groove of DR1 molecules. To further test this interpretation another mutant derived from $\Delta(87-102)$ HA was designed, which carried a Y $\rightarrow$ D substitution (position 95 of the mutant) at the major anchor residue of the HA(306–318) peptide sequence

(corresponding to the 308 position). The Y $\rightarrow$ D substitution is known to reduce drastically the affinity of the HA peptide for DR1 molecules (Jardetzky *et al.*, 1990). The $\Delta(87-102)$ HA Y $\rightarrow$ D mutant, $\Delta(87-102)$ HA and wild-type Ii were compared for their capacity to associate with DR1 α and β chains produced *in vitro* (Figure 2C). SDS-resistant heterotrimers were not detected with $\Delta(87-102)$ HA Y $\rightarrow$ D, indicating that SDS-resistant heterotrimers observed with $\Delta(87-102)$ HA result from binding of the HA sequence in the DR1 peptide-binding groove. For wild-type Ii, we failed to observe SDS-resistant complexes.

These data show that in the HA-containing mutants, the HA region probably binds in the groove of associated class II molecules, thus stabilizing them. We therefore conclude that the location of the CLIP region in the Ii chain allows its access to the peptide-binding site of class II molecules. In contrast, we never observed SDS-resistant

Fig. 3. Peptide binding to HLA-DR1 molecules produced *in vitro*. After co-translation of DR1 α and β chains, microsomes were lysed in a small volume of detergent and divided into equal parts. Each pool was incubated for 16 h on ice in the absence or presence of the indicated peptides at 50 μ M. Immunoprecipitations were carried out with TU36 mAb, from which half of the material was eluted at 95°C (B), the other half at 25°C (NB).

heterotrimers with wild-type Ii, even when using a mild detergent to solubilize microsomes (Figures 2C and 4B; also data not shown), in agreement with previous observations using mouse Ii (Germain and Hendrix, 1991).

CLIP peptides stabilize HLA-DR1 molecules produced *in vitro*

It could be argued that our experimental conditions did not allow wild-type Ii to associate strongly enough through its CLIP region with DR1 molecules to produce SDS-resistant heterotrimers. This seems unlikely as it was previously shown that HA(306–318) as well as CLIP(81–104) were able to stabilize DR1 molecules produced in similar systems (Bijlmakers *et al.*, 1994a; Hedley *et al.*, 1994). However, to rule out this possibility, we analysed a set of synthetic peptides for their capacity to bind and stabilize DR1 molecules produced *in vitro*. DR1 α and β chains were translated together and incubated for 16 h with 50 μ M peptide at 4°C. Class II complexes were immunoprecipitated with the TU36 mAb and eluted in 4% SDS at 25° and 95°C (Figure 3).

As expected, both HA(306–318) and CLIP(90–105) peptides gave rise to SDS-resistant complexes migrating with an apparent mol. wt of 55.5 kDa (Figure 3). Interestingly, CLIP(81–105) generated SDS-resistant complexes running faster with an apparent mol. wt of 53 kDa (Figure 3). This suggests that CLIP(81–89) may induce a conformation different from the one induced by conventional antigenic peptides such as HA(306–318), as previously suggested (Kropshofer *et al.*, 1995a,b). In addition, these data show that, under the conditions used, CLIP associated efficiently with DR1 molecules produced *in vitro*, generating SDS-resistant complexes. Therefore, the absence of $\alpha\beta$ Ii SDS-resistant complexes cannot be ascribed to our experimental conditions, but rather to the inability of the CLIP region to bind in a similar manner to $\alpha\beta$ dimers as the CLIP peptide.

Fig. 4. SDS-resistant $\alpha\beta$ Ii complexes are formed upon the deletion of the 82–89 region of Ii. (A) Association of Ii forms mutated in the 82–89 region with DR1 molecules was tested as follows. The three indicated mutants were translated *in vitro* together with DR1 α and β chains. Detergent-lysed microsomes of these translations were divided into two equal parts and immunoprecipitated with mAbs TU36 and PIN1 (one part each). Half of the protein A- or G-bound material was eluted at 95°C (B), the other half at 25°C (NB). (B) Association of wild-type Ii and of the $\Delta(82-89)$ mutant with DR1 molecules was tested as follows. Wild-type Ii and the $\Delta(82-89)$ mutant were translated *in vitro* together with DR1 α and β chains. Microsomes of these translations were lysed in 1% digitonin, divided into three parts and immunoprecipitated with mAbs TU36, 1B5 and PIN1 (one part each). Half of the protein A- or G-bound material was eluted at 95°C (B), the other half at 25°C (NB).

Deletion of the 82–89 region of Ii allows the formation of $\alpha\beta$ Ii SDS-resistant complexes

It has been shown that the N-terminal portion of CLIP, residues 81–89, reduces the stability of DR1–CLIP complexes (Kropshofer *et al.*, 1995a,b). Our data (Figure 3) also suggest that these residues have an effect on the conformation of DR1. Possibly, this region affects the binding of the CLIP region to the DR1 peptide-binding groove, thus explaining the absence of $\alpha\beta$ Ii SDS-resistant complexes. We therefore investigated the role of this region by deleting aa 82 to 89 or replacing them with other sequences (IR4 and IR5) (see Figure 1A).

All three mutants associated efficiently with DR1 $\alpha\beta$ complexes but the efficiency of association was slightly reduced when compared with wild-type Ii (Figure 4A). This suggests that the 82–89 region contributes to Ii association with DR1 molecules. SDS-resistant heterotrimers were undetectable with $\Delta(82-89)$ IR4 and $\Delta(82-$

Fig. 5. Association of Ii with HLA-DR1 molecules occurs in the absence of the CLIP(87–102) sequence. Association of HLA DR1 molecules translated *in vitro* was tested as follows. (A) With mutants of Ii with internal deletions of the CLIP region. (B and C) With mutants of Ii with IR sequences in place of the CLIP region (see Figure 1A). Wild-type Ii (p33A) and the indicated mutants of Ii were translated *in vitro* together with DR1 α and β chains. Detergent-lysed microsomes of these translations were divided into two equal parts and immunoprecipitated with mAbs TU36 and PIN1 as indicated. Immunoprecipitates were eluted at 95°C.

89)IR5 while they were faintly visible with $\Delta(82-89)$, in material eluted from TU36 at 25°C (Figure 4A, see arrow). When we solubilized the microsomes in 1% digitonin instead of 0.5% NP-40, again no SDS-resistant heterotrimers were observed with wild-type Ii (Figure 4B). However, such complexes (apparent mol. wt 97 kDa) were now more clearly visible with the $\Delta(82-89)$ mutant in material eluted at 25°C from TU36, 1B5 and PIN1 (Figure 4B, see arrow) than when using 0.5% NP-40 (Figure 4A and data not shown). Sequential electrophoresis, as in the experiment presented in Figure 2D, revealed that these complexes were composed of DR1 α and β chains associated with $\Delta(82-89)$ (data not shown). Pulse-chase analysis of COS cells transiently expressing $\Delta(82-89)$ together with DR1 α and β chains gave rise to similar association and formation of SDS-resistant complexes. In addition, we observed that the heterotrimers acquired Endo H-resistant glycans with time, indicating that $\Delta(82-89)$ was exported from the ER and thus correctly folded. Although the SDS-resistant $\alpha\beta\Delta(82-89)$ complexes were present in small proportions, they suggest that the 82–89 region of Ii may indeed affect the binding of the core CLIP region to the DR1 peptide-binding groove. In contrast, this region seems to affect to a lesser extent the binding of the HA(306–318) sequence when part of Ii as the $\Delta(91-102)$ HA mutant, which contains the Ii 81–90 region, was able to form SDS-resistant complexes with DR1 α and β chains (see above).

Two other Ii regions than the core CLIP segment contribute to Ii association with HLA-DR1 molecules

To analyse the remainder of the CLIP region of Ii with regard to the association with DR1 molecules, two other sets of mutants were designed. For the same reasons as stated above in the case of the HA-containing mutants,

we focused again on three parts of the CLIP region: aa 81–102, 87–102 and 91–102. These regions were either deleted or replaced by a sequence (IR1) not expected to bind DR1 molecules (see Figure 1A).

Analysis of the first set of Ii mutants with deletions revealed that association with DR1 molecules did not occur in the absence of residues 81–102, whereas it was slightly detectable when residues 87–102 were deleted and more visible when only residues 91–102 were deleted (Figure 5A, TU36 lanes). All three mutants were produced efficiently, as demonstrated by parallel immunoprecipitation with PIN1 (Figure 5A). These data suggest that residues 81–90 contribute to Ii association with DR1, in agreement with our results obtained with mutated forms of Ii in the 82–89 region (see above).

This conclusion was further supported by the analysis of the second set of Ii mutants with the IR1 sequence in place of the CLIP region (see Figure 1A). It revealed that DR1 molecules associated efficiently with $\Delta(91-102)$ IR1, and less efficiently but still very clearly with $\Delta(87-102)$ IR1, while an extremely weak association, if any, was observed with $\Delta(81-102)$ IR1 (Figure 5B). Note that in the latter mutant the IR1 sequence is five residues shorter than the deleted sequence. This may induce a misplacement of the Ii region, which is C-terminal of CLIP, preventing its interactions with class II molecules. To test this interpretation, we designed the $\Delta(81-102)$ IR1bis mutant which contained the IR1 sequence extended by five aa residues (see Figure 1A). This mutant was found to associate weakly with DR1 molecules (data not shown), suggesting that residues located in the (103–216) region, C-terminal of CLIP, contribute to the association of Ii with DR1 molecules. Such contribution could not take place in the first set of mutants with deletions, which either do not associate or associate only weakly, due to the length of the deletions which may position the region C-terminal

of CLIP too far from its original location. In contrast, introduction of the IR1 sequence in the $\Delta(87-102)$ and $\Delta(91-102)$ mutants may restore such interactions as it enhanced significantly their ability to associate. Finally, association with DR1 molecules was significantly reduced with the $\Delta(87-102)$ IR1 mutant when compared with wild-type Ii (Figure 5A), suggesting that the 87–102 sequence contributes to DR1 association in agreement with previous findings (Freisewinkel *et al.*, 1993; Bijlmakers *et al.*, 1994b; Romagnoli and Germain, 1994).

A summary of the ability of the different Ii mutants to associate with DR1 molecules is presented in Table I. Taken together, our data suggest that three zones of contact exist between Ii luminal domain and DR1: 1) the N-terminal part of CLIP: 81–90; 2) essentially the core CLIP segment: 91–102; and 3) an area located in the region C-terminal of CLIP. Obviously, Ii contains the three zones of contact and therefore has a better capacity to associate than $\Delta(91-102)$ IR1 which contains the 81–90 region and the C-terminal zone, but lacks the core CLIP sequence. Compared with the latter, $\Delta(87-102)$ IR1 has lost only a part of the first region, resulting in a reduced capacity to associate. This capacity was even more reduced in the case of $\Delta(81-102)$ IR1bis which would only interact through the C-terminal region.

To rule out any contribution of the inserted IR1 sequence to the association of $\Delta(91-102)$ IR1 and $\Delta(87-102)$ IR1 with DR1 molecules, two approaches were undertaken. First, four additional mutants were designed, which carried other sequences termed IR2 and IR3 (see Figure 1A) in place of the CLIP region. The IR2 aa sequence was based on a previous study (O'Sullivan *et al.*, 1991b), which described aa substitutions in the HA(306–318) peptide that result in the loss of binding to DR1 molecules. IR3 is the translation product of the oligonucleotide encoding the IR2 peptide inserted in the reverse orientation. The four Ii mutants: $\Delta(91-102)$ IR2, $\Delta(91-102)$ IR3, $\Delta(87-102)$ IR2 and $\Delta(87-102)$ IR3, were found to associate with co-translated DR1 molecules with roughly the same efficiency as the original IR1-containing mutants (Figure 5C).

As a second approach, the IR1 sequence (see Figure 1A) was chemically synthesized. Using an *in vitro* binding assay based on high-performance size-exclusion chromatography (Kropshofer *et al.*, 1993), this IR1 peptide was found unable to compete binding to purified DR1 molecules of an HA(306–318) peptide labelled with the fluorophore AMCA, even at high concentration. In contrast, unlabelled HA(306–318) peptide competed efficiently (data not shown). In addition, the three sequences (IR1, IR2 and IR3) do not show any similarity to the DR1 binding motif (Jardetzky *et al.*, 1990; O'Sullivan *et al.*, 1991a; Hammer *et al.*, 1992, 1993, 1994). Furthermore, none of the Ii mutants either with a deletion or a substitution of the core CLIP region was found capable of generating SDS-resistant heterotrimers. Indeed, elution at 25°C instead of 95°C generated patterns on SDS gels identical to those presented in Figure 5 (data not shown). Thus, association of the Ii mutants with IR sequences in place of the CLIP region is unlikely to result from the ability of these sequences to interact with class II molecules. To check that the substitution introduced in our mutants did not impair their folding, pulse-chase experiments of COS cells transiently expressing $\Delta(91-102)$ IR1

Fig. 6. Inhibition of peptide binding to HLA-DR1 molecules by wild-type and substituted forms of Ii. Wild-type Ii, $\Delta(91-102)$ IR3 and $\Delta(87-102)$ IR3 (see Figure 1A) were translated *in vitro*, together with DR1 α and β chains. Digitonin-lysed microsomes of these translations were first incubated for 16 h on ice with 50 μ M of the HA peptide. Each of them was then divided into two equal parts and immunoprecipitated with mAbs TU36 (noted Tu) and PIN1 (noted Pi). Supernatants from PIN1 were then precleared with protein G beads and subjected to a second immunoprecipitation with TU36 (noted Tu^o). All elutions were carried out at 25°C (noted NB) with the exception of the first lane carried out at 95°C (B).

together with DR1 α and β chains were carried out. Formation of heterotrimers which acquired Endo H-resistant glycans with time was observed, indicating that $\Delta(91-102)$ IR1 was exported from the ER and was correctly folded (data not shown).

We conclude that association of class II molecules with Ii can still take place in the absence of the residues 87–102, provided that the length of Ii is maintained. Our results also show that, in addition to the core CLIP segment, residues from two other Ii regions contribute to the association with DR1 molecules: the 81–90 region and a region C-terminal of the CLIP region.

Inhibition of peptide binding to HLA-DR1 molecules by Ii mutants

One of the important functions of Ii is to prevent peptide binding to class II molecules before they reach the endocytic pathway, where Ii is degraded and class II molecules are able to bind peptides (Wolf and Ploegh, 1995). Studies using truncation mutants of human and mouse Ii concluded that this function, as well as the capacity of Ii to associate with class II molecules, were both dependent on the CLIP region (Bijlmakers *et al.*, 1994b; Romagnoli and Germain, 1994). We analysed our Ii mutants for their capacity to inhibit peptide binding to associated class II molecules.

Translation of α and β chains together with either p33Ii, $\Delta(91-102)$ IR3 or $\Delta(87-102)$ IR3, followed by incubation with the HA peptide (50 μ M) for 16 h at 4°C resulted in a mixture of $\alpha\beta$ -HA and $\alpha\beta$ Ii complexes as judged by immunoprecipitation with TU36 (Figure 6, see Tu lanes). We next examined whether peptide and Ii chains were bound to the same $\alpha\beta$ heterodimers or whether Ii chain-associated and peptide-associated class II molecules were separate populations. Parallel immunoprecipitation with PIN1 recovered Ii chain-associated $\alpha\beta$ complexes but did not remove SDS-resistant $\alpha\beta$ -HA complexes, as judged by their absence from the material eluted from PIN1 at 25°C and by the sequential immunoprecipitation by TU36 (Figure 6, see Pi and Tu^o lanes, respectively). Indeed,

amounts of SDS-resistant complexes present in the TU36 lanes (comparing Tu versus Tu° in Figure 6) were found comparable by phosphorimaging while amounts of β chain were reduced in the Tu° lanes, as expected. In addition, SDS-resistant $\alpha\beta$ -HA complexes were totally undetectable in the material eluted from PIN1 even on long exposures. Note that these experiments were performed under mild conditions of solubilization to maximize the chance of detecting such complexes. Similar results were obtained with the following mutants: $\Delta(87-102)$ IR1 and 2, $\Delta(91-102)$ IR1 and 2, $\Delta(87-102)$ HA, $\Delta(82-89)$, $\Delta(82-89)$ IR4 and 5 (data not shown).

These results show therefore that $\Delta(91-102)$ IR and $\Delta(87-102)$ IR behaved like wild-type p33Ii, in terms of inhibition of peptide binding to DR1. This strongly suggests that the CLIP(87-102) sequence is not required for Ii inhibition of peptide binding to DR1 molecules.

Discussion

Since the crystal structure of a HLA-DR3-CLIP complex was solved, demonstrating that the core CLIP(89-101) peptide binds essentially like a conventional antigenic peptide (Ghosh *et al.*, 1995), the question has been raised whether the CLIP region of intact Ii has access to the peptide-binding site of class II molecules. We show here that mutants of Ii with overlapping parts of the CLIP region exchanged for the sequence of the DR1 binding HA(306-318) peptide formed SDS-resistant heterotrimers with DR1 α and β chains translated *in vitro*. Similar results were obtained *in vivo* for one of these mutants. Substitution of the major anchor residue (Y→D) within the HA sequence of one of these Ii mutants abolished its capacity to form SDS-resistant heterotrimers, but not its ability to associate with DR1 molecules. Although the biochemical basis of resistance to dissociation by SDS remains to be elucidated, acquisition of such resistance implies conformational changes and has thus far only been associated with binding of peptides to the class II peptide-binding groove. Our data strongly suggest that the HA sequence in place of the CLIP region of the Ii mutants tightly binds to the DR1 peptide-binding site. Therefore, the location of the CLIP region within Ii seems compatible with its access to the peptide-binding site of DR1 molecules.

SDS-resistant heterotrimers were not detectable with wild-type Ii, even under mild conditions of solubilization. However, in our experimental system CLIP peptides as well as the HA(306-318) peptide were able to bind to DR1 $\alpha\beta$ chains and to form SDS-resistant complexes as previously shown (Bijlmakers *et al.*, 1994a; Hedley *et al.*, 1994). In addition, CLIP and HA(306-318) possess comparable affinities for DR1 molecules (Sette *et al.*, 1995). If the core CLIP region of Ii has access to the class II groove, why would SDS-resistant $\alpha\beta$ Ii not be observed? Several arguments indicate that this phenomenon might be due to the proline-rich region (81-90) of Ii. The CLIP(81-89) peptide has been shown to reduce the half-life of DR molecules complexed to various peptides, including CLIP(90-105) (Kropshofer *et al.*, 1995b). Electrophoretic mobility of DR1 complexes formed with either CLIP(81-105) or CLIP(90-105), suggested that these complexes may differ in their conformation since

complexes with CLIP(81-105) had a faster mobility (Figure 3). We also show that an Ii mutant deleted in aa 82-89, termed $\Delta(82-89)$, was able to form SDS-resistant heterotrimers with DR1 $\alpha\beta$ chains, suggesting that the core CLIP region of this mutant associated tightly to the DR1 peptide-binding groove. Taken together, these data indicate that the 81-90 region causes the core CLIP region to bind differently to DR1 molecules than the CLIP peptide. Considering that the 81-90 region binds to class II molecules (see below) it can be proposed that these residues impose physical constraints upon the adjacent core CLIP segment. These constraints may allow only part of the interactions that take place in the DR-CLIP complexes (Ghosh *et al.*, 1995) as previously suggested from peptide-binding studies (Kropshofer *et al.*, 1995a). Crosslinking of α 1 and β 1 domains by the core CLIP region of associated Ii would thus remain incomplete resulting in SDS-instability.

Despite the presence of aa 81-90, the $\Delta(91-102)$ HA mutant (where the deleted sequence has been replaced by the HA sequence) associated efficiently with DR1 molecules and formed SDS-resistant heterotrimers. However, $\Delta(91-102)$ HA formed SDS-resistant complexes with a lower efficiency than $\Delta(87-102)$ HA, where the 81-90 region is incomplete. This may suggest that: (i) the 81-90 region within the $\Delta(91-102)$ HA mutant can still affect the binding of the adjacent HA region to the DR1 binding groove; and (ii) removal of the 87-90 aa (which contains one proline residue) within the $\Delta(87-102)$ HA mutant allows a better stabilization of the DR1 complexes through interaction with the HA region. It is noteworthy that CLIP(81-89), which is able to reduce the half-life of various class II-peptide complexes, had no effect on the half-life of the highly stable complexes; DR3-MOMP(246-260), DR3-HSP65(3-13) and especially DR1-HA(306-318) (Kropshofer *et al.*, 1995b; H.Kropshofer, personal communication). Considering full-length Ii, the constraints imposed by the 81-90 region can be overcome either by replacing the core CLIP region by the sequence of the HA peptide which binds DR1 molecules more stably than does the CLIP peptide, or by deleting the aa 82-89 [as $\Delta(91-102)$ HA and $\Delta(82-89)$, respectively form SDS-resistant complexes with DR1]. Therefore, stability of the interaction of the sequence potentially binding to the DR1 groove and accessibility of this sequence to the DR1 groove seem to govern the ability to form SDS-resistant complexes. Our data suggest that in wild-type Ii the 81-90 region creates an inappropriate spacing for the complete access of the core CLIP region to the DR1 groove. Given the fact that aa 81-90 contain four proline residues, it can be proposed that the 81-90 aa could adopt a hook-like structure. If this were the case, it would seem likely that the folding of IR4 and 5 sequences used to replace aa 81-90 (see Figure 1A) did not generate an appropriate spacing either, as such replacement did not result in a capacity to generate SDS-resistant forms. Note that this feature of the 81-90 region may only concern a subset of the DR allelic products. Given the high polymorphism of the DR molecules which is known to influence the affinity of the core CLIP peptide, the Ii 81-90 region may vary in its contribution to Ii binding to DR chains and in the way it affects the binding of the core CLIP region. Indeed, we have observed SDS-resistant

$\alpha\beta$ Ii complexes in pulse-chase experiments performed with a DR10/DR11 cell line. Such complexes appear at 1 h of chase and then disappear while SDS-resistant $\alpha\beta$ -peptide complexes start to accumulate with time (P.Stumptner and P.Benaroch, unpublished data). Finally, cleavage occurring *in vivo*—notably at positions 81–83 of Ii once $\alpha\beta$ Ii reaches the endocytic pathway (Rudensky *et al.*, 1991; Chicz *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992)—may release the constraints imposed by the 81–90 region. This, possibly in conjunction with low pH, would allow tight binding of the core CLIP to the class II groove as observed in the DR3–CLIP crystal (Ghosh *et al.*, 1995).

Although all class II allele products associate with Ii, which inhibits premature peptide binding (see Germain *et al.*, 1996), their affinity for CLIP peptides varies by several orders of magnitude (Avva and Cresswell, 1994; Sette *et al.*, 1995). This suggests that areas other than the CLIP region are involved in the association with class II and that the role of the CLIP region is not central for the functions exerted by Ii. We show here that replacement of the CLIP (87–102) and (91–102) region by different sequences (termed IR1 to 3) results in mutants still able to associate with class II molecules. Putative contribution of the inserted sequences to the association was ruled out by several means. First, we obtained the same results using three different sequences for CLIP replacement. Second, none of the three sequences shared the DR1 binding motif (Jardetzky *et al.*, 1990; O'Sullivan *et al.*, 1991a; Hammer *et al.*, 1992, 1993, 1994). Third, competitive binding studies performed with a synthetic peptide corresponding to one of these inserted sequences demonstrated its inability to bind DR1 molecules. Our data thus confirm recent findings performed with Ii-derived peptides and recombinant or proteolytic Ii fragments showing that other region(s) of Ii besides CLIP contribute to the association with class II molecules (Adams and Humphreys, 1995; Park *et al.*, 1995; Vogt *et al.*, 1995; Newcomb *et al.*, 1996).

Indeed, our results define two additional areas on Ii, located on each side of the core CLIP region, which are involved in association with DR1 molecules. The first region (81–90) was identified by showing that $\Delta(81-102)$ was unable to associate with DR1 molecules, while $\Delta(87-102)$ associated weakly and $\Delta(91-102)$ slightly better (see Figure 5 and Table I). Accordingly, deletion or replacement of the 82–89 region led to a slightly reduced capacity of association with DR1 when compared with wild-type Ii. The existence of the second region was deduced from the following results: insertion of the IR1, 2 or 3 sequence into the $\Delta(87-102)$ and $\Delta(91-102)$ mutants enhanced their capacity to associate with DR1 (see Figure 5 and Table I). This suggests that deletion of aa 87–102 or 90–102 prevents interactions with DR1 molecules by misplacement of a region C-terminal of CLIP, while insertion of the IR sequences restored these interactions. This conclusion is supported by the fact that $\Delta(81-102)$ IR1bis, which has the same length as wild-type p33Ii, associated with DR1 in contrast to $\Delta(81-102)$ IR1, which did not associate and in which the IR1 sequence is five residues shorter than the deleted one. Finally, the $\Delta(87-102)$ IR1 mutant, which is only one residue longer than wild-type Ii, clearly associated less efficiently than Ii, suggesting

that the 87–102 region contributes significantly to DR1 association, in agreement with previous conclusions (Freisewinkel *et al.*, 1993; Bijlmakers *et al.*, 1994b; Romagnoli and Germain, 1994).

Together, our data indicate that Ii luminal domain contains at least the three following zones of contacts with DR1 molecules: 1) the N-terminal part of CLIP: 81–90; 2) essentially the core CLIP segment: 91–102; and 3) an area located in the 103–216 region C-terminal of CLIP. Mutation in one of these three areas does not significantly affect association with DR1 molecules, while abrogation of contact through two of these three areas leads to a more reduced association (see Table I). In contrast to our results, the truncation Ii1–96, in which the first and part of the second contact regions are present, was reported to be devoid of capacity to bind to DR1 (Bijlmakers *et al.*, 1994b). However, a weak association may have occurred with this truncation as DR1 α and β chains are clearly visible, in addition to the mutant, on the published figure in the immunoprecipitate performed with an anti-Ii mAb (Bijlmakers *et al.*, 1994b). Other previous results obtained with truncated forms of Ii or Ii mutants with internal deletions are in agreement with our proposal (Freisewinkel *et al.*, 1993; Bijlmakers *et al.*, 1994b; Romagnoli and Germain, 1994). Finally, further contact between class II and Ii may be mediated by the transmembrane domains (Amigorena *et al.*, 1995; Newcomb *et al.*, 1996).

One important property of Ii is inhibition of class II binding to peptides and unfolded proteins before class II complexes reach the endocytic loading compartments. Testing our mutants for this capacity, we found that the $\Delta(91-102)$ IR and the $\Delta(87-102)$ IR mutants, which are able to associate with DR1 molecules, behave like wild-type Ii and thus appear also to be able to prevent peptide binding to associated DR1 molecules. This strongly suggests that binding of the core CLIP region in the groove of associated class II molecules is not an absolute requirement for peptide binding inhibition. This is consistent with our proposal that the core CLIP region of wild-type Ii is affected in its binding to the DR1 groove. Interaction of Ii with class II molecules through the 81–90 contact region may promote a conformation of class II in which they would be unable to bind to peptides. In support of this hypothesis, we already mentioned that peptides containing this region have been shown to promote the dissociation of antigenic peptides bound to class II (Adams and Humphreys, 1995; Kropshofer *et al.*, 1995b). A similar role for the region C-terminal of CLIP by itself appears unlikely since the ectodomain 118–208 of Ii, which would contain this region, was found to associate with DR1 molecules and to promote rather than to inhibit peptide binding to DR1 molecules (Park *et al.*, 1995). Overall, our data are compatible with the hypothesis that the 81–90 region either by itself or in conjunction with other Ii regions, alters the conformation of class II so as to prevent peptide binding.

In summary, our results show that the 81–90 region: 1) contributes to the association of Ii with class II molecules; 2) seems to affect binding of the core CLIP region to the DR1 peptide binding site; and 3) may promote a class II conformation different from that in which these molecules accommodate classical antigenic peptides. It could also be proposed from our data that flanking regions of CLIP,

which associate with class II molecules, position the core CLIP segment just above the groove of associated class II molecules, in agreement with a recently proposed model (Park *et al.*, 1995; Cresswell, 1996). Providing that the sequence used to replace the core CLIP region does not modify the conformation adopted by the $\alpha\beta$ Ii complex, any polypeptide in place of CLIP should preclude access of antigenic peptides to the class II groove. This in turn would explain how class II allele products with very low affinity for CLIP can still associate with Ii and be unable to bind peptides prematurely. Finally, replacement of the core CLIP region by antigenic peptide sequences as in our HA-containing mutants, may represent a powerful approach to load endogenously class II molecules with chosen peptides.

Materials and methods

DNA constructs

cDNAs encoding the human p33 Ii (Claesson *et al.*, 1983), the HLA-DR1 α (Larhammer *et al.*, 1982) and β chains (Bell *et al.*, 1985) were cloned into pSP72 (Promega) behind the T7-RNA polymerase promoter and were described previously (Bijlmakers *et al.*, 1994a). The plasmid p33A, which contains Ii cDNA, has been used before for *in vitro* translation and gave two translation products of close molecular weight, both with two N-linked carbohydrate chains. This was interpreted as the result of alternative initiation of translation at methionine at position 16 (Bijlmakers *et al.*, 1994a,b). However, in sequencing the p33A plasmid, we found an additional methionine codon located upstream of the first one at position -13. This codon is in frame and does not share the Kozak consensus sequence. Sub-cloning of only the coding sequence for p33 Ii was therefore carried out using PCR to create *Eco*RI and *Xho*I sites which were used for insertion into pSP72, resulting in a plasmid designated p33B. *In vitro* transcription and translation performed with p33B gave rise to a single fully glycosylated product which corresponded to the lower band (33 kDa) of the two obtained with p33A (34.5 and 33 kDa) (not shown). Therefore it seems likely that in p33A, due to the suboptimal context of the ATG in position -13, some 40S ribosomal subunits bypass this ATG and initiate instead at the second one in position 1, as already observed in other situations (Kozak, 1991). Experiments were carried out with the p33B plasmid with one exception shown in Figure 5 which was achieved with p33A. However, similar results have been obtained with the p33A and p33B plasmids for Ii association to DR1 molecules and for Ii capacity to inhibit peptide binding (see Bijlmakers *et al.*, 1994a; Figures 2C, 4B and 6; also data not shown).

Mutants of Ii were created using a PCR-based approach. All PCR amplifications were carried out with PFU polymerase (Stratagene). All constructs were cloned downstream of the T7 polymerase promoter, the first ATG being 47 bp downstream of the transcription start and possessing the Kozak consensus sequence (Kozak, 1991). All constructs carry a stop codon immediately after the last codon of Ii (position 216) and were checked by automated DNA sequencing kindly performed by the team running the DNA sequencing facility at the ICGM. The length in aa of the polypeptides encoded by the different constructs is indicated in Table I. A first set of Ii mutants with internal deletions was created by a two-step procedure. First, we amplified the Ii fragment corresponding to residues 1 to either 80, 86 or 90 by PCR, creating at their 5' and 3' ends an *Eco*RI site and a *Kpn*I site, respectively. Second, the cDNA fragment corresponding to residues 103 to 216 was amplified by PCR creating at the 5' and 3' ends a *Kpn*I site and a *Xba*I site, respectively. Then, the latter fragment plus each of the three former were used as templates to generate, by PCR, fusion products encoding for the three mutants with an internal deletion from 81 to 102, 87 to 102 and 91 to 102, respectively (see Figure 1A). Following an *Eco*RI and *Xba*I digestion, these PCR products were cloned into the corresponding sites of pSP72. These constructs contained a *Kpn*I site in place of the deleted sequences. A second set of mutants was generated using this *Kpn*I site to insert double-strand oligonucleotides flanked at each end by a half-*Kpn*I site and encoding for the HA, IR1, IR2, and IR3 sequences (see Figure 1A). Note that IR1 and IR3 are respectively the translation products of the oligonucleotides encoding for HA and IR2 inserted in the reverse orientation.

The $\Delta(87-102)$ HA Y $\rightarrow$ D mutant was generated using the $\Delta(87-102)$ HA mutant as a template to introduce by PCR a T $\rightarrow$ G substitution in the HA coding sequence resulting in a Y $\rightarrow$ D substitution in position 95 of the polypeptide. The PCR fragment was then digested by *Kpn*I and inserted into the *Kpn*I site of the $\Delta(87-102)$ construct.

The $\Delta(81-102)$ IR1bis mutant was generated using the $\Delta(81-102)$ IR1 construct as a template to amplify by PCR the region containing the IR1 sequence by means of a 5' oligonucleotide designed to create in its 5' overhanging sequence a new *Kpn*I site followed by three additional codons and a mutation in the 5' *Kpn*I site of the $\Delta(81-102)$ IR1 construct. Digestion of the PCR product resulted thus in a DNA fragment encoding the IR1bis sequence (see Figure 1A) flanked by half-*Kpn*I sites. This fragment was then inserted in the *Kpn*I site of the $\Delta(81-102)$ mutant, resulting in the $\Delta(81-102)$ IR1bis construct.

Another set of mutants concerning the 82-89 region of Ii was designed as follows. First, two Ii DNA fragments encoding for aa 1-81 and 90-216 were amplified by PCR. Oligonucleotides were designed in order to create an *Eco*RI site at the 5' end of the first PCR product, a *Hind*III site and a *Xho*I site at the 5' and 3' ends of the second product, respectively. Note that the cDNA of wild type Ii contains a *Hind*III site corresponding to residues 80 and 81. The first PCR product was digested by *Eco*RI and *Hind*III, while the second product was digested by *Hind*III and *Xho*I. They were ligated together into the *Eco*RI and *Xho*I sites of pSP72, resulting in the $\Delta(82-89)$ construct. The internal *Hind*III site was then used to insert double-strand oligonucleotides encoding for IR4 or IR5 sequences flanked by half-*Hind*III sites (see Figure 1A). Note that IR5 is the translation product of the oligonucleotides encoding for IR4 inserted in the reverse orientation.

Antibodies, peptides

The following mouse mAbs were used: TU36 (Shaw *et al.*, 1985), which is anti HLA-DR $\alpha\beta$, 1B5 (Adams *et al.*, 1983), which is anti HLA-DR α , PIN1 (Lamb and Cresswell, 1992), which is specific for the N-terminus of human Ii and was kindly provided by Dr S.Amigorena and BU45 (Wright *et al.*, 1990) directed against the C-terminus of human Ii (The Binding Site). The HLA-DR1 presentable peptides from influenza virus haemagglutinin HA(306-318) (Rothbard *et al.*, 1988) and human Ii chain CLIP(89-105) and CLIP(81-105) (Chicz *et al.*, 1992) were a generous gift of Dr H.Kropshofer. The peptide corresponding to the IR1 sequence (see Figure 1A) was synthesized by Neosystem (France).

In vitro transcription and in vitro translation

Transcriptions were performed using T7 RNA polymerase following instructions given by the manufacturer (Promega). RNA was stored in 48% isopropyl alcohol. The optimal amount of RNA for translation was determined empirically for each separate batch of RNA.

In vitro translations were performed for 60 min at 30°C with rabbit reticulocyte lysate, (FlexiTM, Promega), in the presence of dog pancreas microsomes (Promega). Typical translation reactions contained 17.5 μ l rabbit reticulocyte lysate, 1.5 μ l dog pancreas microsomes, 0.625 μ l 10 mM oxidized glutathione, 0.8 μ l 2.5 M KCl, 0.5 μ l 1 mM amino acid mixture minus methionine, 2.5 μ l [³⁵S]methionine (Dupont, 10 mCi/ml) and 1-3 μ l mRNA dissolved in H₂O (total reaction volume 25 μ l).

Immunoprecipitations

After translation, microsomes were pelleted by centrifugation at 15 000 r.p.m. for 5 min at 4°C and subsequently lysed in 1 ml NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂) supplemented with 1 mM PMSF. All subsequent incubations were performed at 4°C for 90 min under constant agitation. Non-specifically bound proteins were removed by pre-clearing once with 2 μ l normal rabbit serum, 1 μ l normal mouse serum and 150 μ l of 10% v/v Zysorbin (Zymed) and once with 150 μ l protein A-Sepharose beads of a 15% slurry. mAbs (400 μ l of hybridoma culture supernatant) were pre-bound to protein A- or protein G-Sepharose beads (150 μ l of a 15% slurry). Beads were then washed before being incubated with pre-cleared lysates. After incubation, pelleted beads were washed four times in 50 mM Tris-HCl, pH 7.4 buffer containing 0.5% NP-40, 150 mM NaCl and 5 mM EDTA. Bound immunocomplexes were eluted in reducing SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% β -mercaptoethanol, 4% SDS) for 5 min at 95°C and subjected to SDS-polyacrylamide gel electrophoresis. To detect 'SDS-resistant' class II complexes, protein A or G pellets were extracted for 15 min with SDS-sample buffer at 25°C before gel electrophoresis. In the experiments described in Figures 4B and 6, solubilization of the microsomes was achieved in PBS containing 1% digitonin (Wako) and subsequent washes of immunocomplexes were carried out in PBS containing 0.5% digitonin. Endoglycosidase H (endo

H) digestions and gel electrophoresis were performed as previously described (Bijlmakers *et al.*, 1994a).

Assay of the ability of the different Ii mutants to associate with DR1 molecules

The ability of the different mutants to associate with DR1 molecules was assayed by co-translation with DR1 α and β chains, followed by immunoprecipitations as described above. Association of $\alpha\beta$ Ii heterotrimers occurs only if the three polypeptides are translocated together in the same microsomes (Bijlmakers *et al.*, 1994a). The proportion of microsomes containing the three chains varies from experiment to experiment. In addition, efficiency of translation can vary from reaction to reaction (despite titration of each separate batch of mRNA). Therefore, gels were analysed by phosphorimager (Molecular Dynamics) or by densitometry and the efficiency of association with DR1 molecules was evaluated by calculation of the ratio of Ii mutant versus DR1 β or α chain present in the complexes eluted from TU36 at 95°C. We took into account background values and methionine contents of the different polypeptides, which are 3, 2 and 14 for α , β and Ii, respectively. The Ii mutants contain 11 methionines except for the $\Delta(82-89)$ series, which have 14. For each independent experiment, results were normalized with regard to the capacity of Ii to associate with DR1. Data from repeats of the experiments are summarized in Table I.

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