

## Mapping Functional Regions in the Luminal Domain of the Class II-associated Invariant Chain

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### Summary

The MHC class II-associated invariant chain interacts in trimeric form with class II molecules, inhibits peptide binding, and mediates targeting of class II molecules to endosomal compartments. To dissect the different functions of the invariant (Ii) chain, a set of cDNAs, encoding truncated forms of the Ii chain, was constructed. mRNAs, transcribed from these cDNAs were translated in vitro, together with mRNAs encoding class II HLA DR1  $\alpha$  and  $\beta$  subunits. An Ii chain truncation that contains the 104 NH<sub>2</sub>-terminal amino acids was able to associate with class II molecules. This construct contains the region from which class II-associated Ii chain peptides (CLIP, amino acids 81–104) are derived. The absence of a further eight residues at the COOH terminus results in a construct of 96 amino acids that is unable to associate with class II molecules. Association of the truncated Ii chains with class II molecules showed a strict correlation with inhibition of peptide binding. Removal of the NH<sub>2</sub>-terminal cytoplasmic tail and transmembrane region of Ii chain and its replacement with a cleavable signal sequence led to aberrant folding and impaired association with class II molecules. The region between amino acids 163 and 183 was found to be essential for visualization of Ii chain homotrimers by covalent cross-linking.

MHC class II molecules are expressed at the cell surface where they present peptides to T cell receptors of CD4<sup>+</sup> cells. Class II molecules are composed of two MHC-encoded subunits, the  $\alpha$  and  $\beta$  chain, which associate in the endoplasmic reticulum (ER)<sup>1</sup> with a third protein, the invariant (Ii) chain. This association is rapid and efficient, due to a large molar excess of the Ii chain in cells expressing class II molecules (1, 2). Ii chains assemble into homotrimers in the ER (3), and form higher order structures upon association with  $\alpha$  and  $\beta$  chains (4, 5). These oligomers consist of Ii chain homotrimers with one, two, or three  $\alpha/\beta$  heterodimers attached.

The Ii chain is a type II membrane protein with a non-cleavable signal anchor sequence. The NH<sub>2</sub>-terminal tail is exposed in the cytosol, while the COOH-terminal bulk of the protein forms the luminal domain (6). Alternative splicing and the use of different translation initiation sites lead to at least four different forms of Ii chain in humans, with a  $M_r$  of 33, 35, 43, 45 kD, respectively (7), of which the 33-kD form (p33) is the most abundant. The protein is modified cotranslationally by N-linked and posttranslationally by O-linked glycosylation (6, 8, 9).

Four functions have been assigned to the Ii chain: (a) association with class II molecules and facilitation of egress of class II out of the ER (10–12); (b) homotrimerization to generate a scaffold onto which  $\alpha/\beta$  heterodimers assemble (4, 5); (c) inhibition of peptide binding by class II molecules (13, 14); and (d) targeting class II molecules to the endocytic pathway (15, 16). In the endosomal compartments, the Ii chain is degraded to allow subsequent binding of peptide (17). Two of the functions have been attributed to specific regions of the Ii chain. A sequence in the cytoplasmic tail functions as a signal to target  $\alpha/\beta$ -Ii complexes to endosomal compartments (15, 16). The region between amino acids 87 and 110 of the mouse Ii chain (which corresponds to the same positions in the human p33 Ii chain) is essential for association with class II molecules (18).

Peptides derived from Ii chain have been eluted from both mouse and human class II molecules (19–22). These peptides, designated class II-associated Ii chain peptides (CLIP), vary in length between 14 and 25 amino acids. They originate from the region between amino acids 81 and 104 in human p33 and mouse Ii chain. Their discovery led to the speculation that (a) Ii chains interact with class II  $\alpha/\beta$  heterodimers via the CLIP region and (b) that this interaction occurs in the peptide binding groove and thereby prevents peptide binding of class II molecules.

We investigated domain-function relationships of the Ii

<sup>1</sup> Abbreviations used in this paper: CLIP, class II-associated Ii chain peptides; DSP, dithiobis (succinimidylpropionate); endo H, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin; Ii, invariant.

chain by *in vitro* translation of truncated Ii chains and class II  $\alpha$  and  $\beta$  chains. We show that amino acids 96–104, located in the CLIP region, are essential for interaction with class II  $\alpha$  and  $\beta$  chains *in vitro*. The ability of Ii chain truncations to inhibit peptide binding coincided with their ability to associate with class II molecules. The region between amino acid 163 and 183 was found to be required for the visualization of Ii chain homotrimers by covalent cross-linking.

## Materials and Methods

**DNA Constructs.** cDNAs encoding the HLA-DR1  $\alpha$  (23) and  $\beta$  chains (24) were kindly provided by Dr. J. Trowsdale (Imperial Cancer Research Fund, London, UK). cDNA encoding the p33 Ii chain (6) was kindly provided by Dr. B. Dobberstein (Zentrum für Molekular Biologie Heidelberg, Heidelberg, Germany). The cDNA encodes the “long” form of Ii, referred to by others as p35. The sequence encoding the cleavable signal peptide of the class II  $\beta$  chain was exchanged for the sequence encoding the signal peptide of mouse class I heavy chain H2-K<sup>b</sup>, to obtain approximately equal translation and translocation efficiencies for  $\alpha$  and  $\beta$  chains. All cDNAs were cloned into pSP72 (Promega Corp., Madison, WI) behind the T7-RNA polymerase promoter.

mRNAs encoding COOH-terminal truncations of the Ii chain were generated in two different ways. 163C, 123C, and 104C were generated by restriction enzyme digestion of the full-length Ii chain cDNA with DrdI, Avall, and NcoI, respectively, which cut in the coding sequence of Ii chain. Plasmids linearized with these enzymes were used for the transcription of mRNA. 183C, 153C, 96C, and 80C were generated by introduction of a BamHI site (by PCR with the enzyme PFU [Stratagene, La Jolla, CA]) after the codons encoding amino acid 183, 153, 96, and 80, respectively. PCR products were digested with EcoRI and BamHI and subcloned in pSP72 (Promega Corp.). After linearization with BamHI, mRNA was transcribed from these cDNAs.

cDNAs encoding NH<sub>2</sub>-terminally truncated Ii chains, fused to the signal peptide of H2-K<sup>b</sup>, were generated by two-step PCR reactions (using the enzyme PFU). Two reactions were performed in the first step. In one reaction Ii chain fragments were generated, using a 5' primer with an overhanging sequence complementary to the H2-K<sup>b</sup> signal peptide cDNA. In the other reaction an H2-K<sup>b</sup> fragment was generated, using a 3' primer with an overhanging sequence complementary to Ii chain cDNA. In the second step, PCR fragments of both reactions were annealed and a fusion product of both cDNA fragments was synthesized by PCR.

**Antibodies and Peptides.** The following anti-HLA-DR1 antibodies were used: the mouse mAb Tü36 (25), a generous gift of Dr. Ziegler (Frieie Universäte, Berlin, Germany), the mouse mAb LB3.1, kindly provided by Dr. J. Strominger (Harvard University, Cambridge, MA). Two Ii chain-reactive mouse mAbs were used: VicY1 (26), which is specific for the NH<sub>2</sub> terminus and was kindly provided by Dr. W. Knapp (University of Vienna, Vienna, Austria) and Bü45 (27) directed against the COOH terminus (from The Binding Site, Ltd., Birmingham, UK). The HLA-DR1 presentable peptide from influenza virus hemagglutinin (HA) (306–318) (28) was a generous gift of Dr. L. Stern (Harvard University). Peptides were dissolved in H<sub>2</sub>O and stored at –20°C.

**Gel Electrophoresis.** SDS-glycine gel electrophoresis were performed as described (29). SDS-glycine gels were fluorographed using dimethylsulfoxide–diphenyloxazole (DMSO-PPO) and exposed to Kodak XAR-5 films.

**In Vitro Transcription and In Vitro Translation.** Transcriptions were

performed using T7 RNA polymerase. RNA was stored in 70% ethanol. The optimal amount of RNA for translation was determined empirically for each separate batch of RNA.

*In vitro* translations were performed with rabbit reticulocyte lysate, not supplemented with dithiothreitol (Flexi™; Promega Corp.), in the presence of dog pancreas microsomes (Promega Corp.). Translations were performed for 60 min at 30°C. Typical translation reactions contained 17.5  $\mu$ l rabbit reticulocyte lysate, 1.5  $\mu$ l dog pancreas microsomes, 0.8  $\mu$ l 2.5 M KCl, 0.5  $\mu$ l 10 mM amino acid mixture minus methionine, and 2.5  $\mu$ l [<sup>35</sup>S]methionine (10 mCi/ml; DuPont, Wilmington, DE), 1–3  $\mu$ l mRNA dissolved in diethylpyrrocarbonate-treated H<sub>2</sub>O (total reaction volume 25  $\mu$ l).

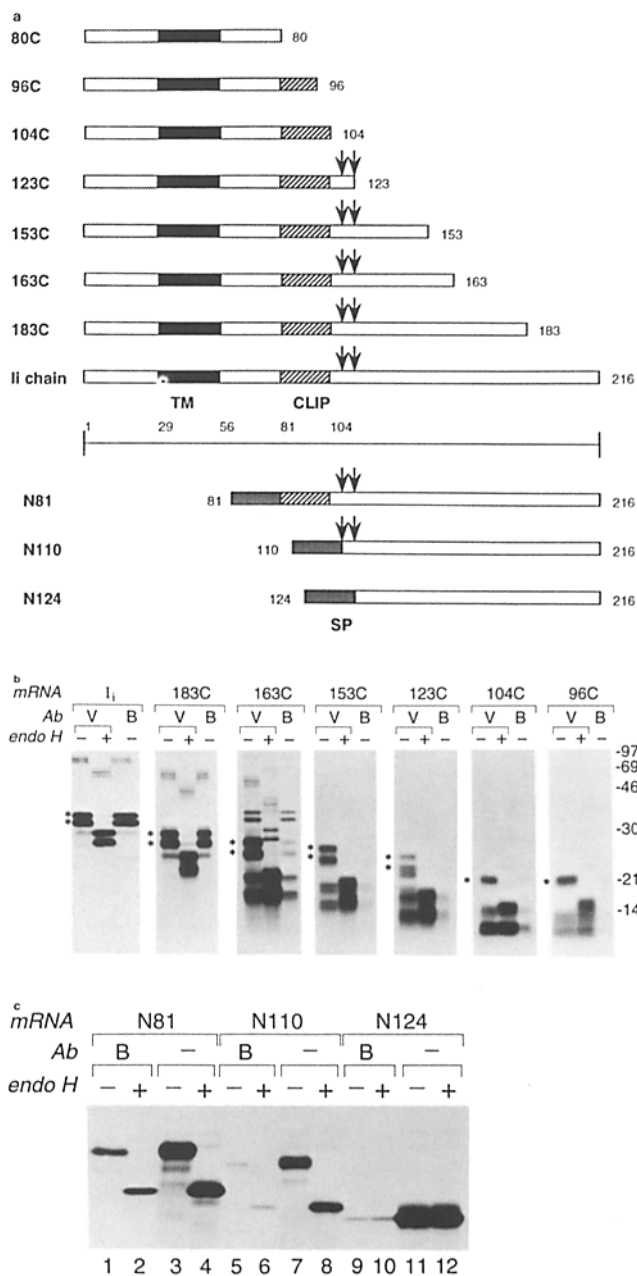
**Immunoprecipitation.** After translation, microsomes were pelleted by centrifugation at 14,000 rpm for 10 min at 4°C and subsequently lysed in 200  $\mu$ l NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl<sub>2</sub>). Solubilized microsomes were either subjected to immunoprecipitations immediately or incubated on ice in the presence of peptides as described in the figure legends. Before immunoprecipitations, the volume was increased to 1 ml by the addition of NP-40 lysis buffer. Nonspecifically bound proteins were removed by preclearing twice with 3  $\mu$ l normal rabbit serum for 45 min and 50  $\mu$ l formaline fixed, heat-killed *Staphylococcus aureus* Cowan strain bacteria (Staph.A; 10% vol/vol) for 30 min. Immunoprecipitations were performed with 2  $\mu$ l ascites or polyclonal serum for 1 h on ice. Immune complexes were removed by adsorption onto 50  $\mu$ l Staph.A. Pelleted bacteria were washed four times in buffer containing 0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA. Bound immune complexes were eluted in reducing SDS-sample buffer for 5 min at 95°C and subjected to SDS-polyacrylamide gel electrophoresis. To detect “SDS-stable” class II  $\alpha/\beta$  heterodimers, Staph.A pellets were extracted for 20 min with SDS-sample buffer (4% SDS final concentration) at room temperature before gel electrophoresis.

**Endo H Digestion.** For endoglycosidase H (endo H) digestion, immune complexes adsorbed to Staph.A were divided into two equal portions. Both portions were denatured in 0.5% SDS, 1%  $\beta$ -mercaptoethanol for 5 min at 95°C. After cooling on ice, reaction buffer (50 mM sodium citrate, pH 5.5) was added to every sample and recombinant endo H (New England Biolabs Inc., Beverly, MA) was added to one half of the samples at a final concentration of 50,000 U/ml. The remaining half of the samples served as mock digestion control. All samples were incubated at 37°C for 60 min.

**Cross-linking with DSP.** For cross-linking experiments, microsomes were lysed in 0.5% NP-40 in PBS containing the cleavable homobifunctional cross-linker dithiobis (succinimidylpropionate) (DSP) (Pierce, Rockford, IL), at the concentrations indicated in the figure legends. DSP was stored in DMSO at a concentration of 10 mg/ml. After incubation for 30 min on ice, glycine was added to a final concentration of 250 mM to quench the cross-linking. After 30 min on ice, aggregates were removed by centrifugation at 14,000 rpm at 4°C for 20 min. Immunoprecipitations were performed as described above. Staph.A-bound immune complexes were eluted in nonreducing SDS-sample buffer.

## Results

**Characterization of Truncated Ii Chains Translated In Vitro.** Deletions at the 3' or 5' end of the human Ii chain cDNA were made as described in Materials and Methods. The proteins encoded by these cDNAs have deletions of various lengths at the COOH or NH<sub>2</sub> termini of the p33 Ii chain (Fig. 1 a). The constructs are designated by the position of their first



**Figure 1.** The Ii chain truncations. (a) Schematic representation of the full-length, the NH<sub>2</sub>-terminally, and COOH-terminally truncated Ii chains. Truncations were made as described in Materials and Methods. The position of the last amino acids of the COOH-terminal truncations are indicated on the right, the position of the first amino acids of the NH<sub>2</sub>-terminal truncations are indicated on the left. Further indicated are: the transmembrane region (TM), the region from which CLIP are derived (CLIP), the glycosylation sites (arrows), and the signal peptide of H2-K<sup>b</sup> (SP). (b) mRNAs encoding the full-length Ii chain (Ii) or COOH-terminally truncated forms of the invariant chain were translated in vitro in the presence of microsomes. Microsome-associated products were subjected to immunoprecipitation with mAb Vic-Y1 (V), against the NH<sub>2</sub> terminus or Bü45 (B), against the COOH terminus. Polypeptides recovered with Vic-Y1 were either mock digested (-) or digested (+) with endo H to remove N-linked carbohydrate chains. Glycosylated polypeptides are indicated (asterisk). Immunoprecipitates were analyzed on reducing 12.5% polyacrylamide gels. Dimers of full-length Ii chain, 183C, and 163C were detected. These are formed by interchain disulfide bridge formation between the

(for the NH<sub>2</sub>-terminal truncations) or last (for the COOH-terminal truncations) amino acid. Thus, 183C encodes amino acids 1-183 and N81 contains amino acids 81-216. mRNAs were transcribed from these cDNAs and translated in vitro using rabbit reticulocyte lysate in the presence of microsomes as ER equivalents. The NH<sub>2</sub>-terminally truncated forms, which do not contain the Ii chain signal peptide, were fused with the cleavable signal peptide of mouse MHC class I heavy chain H2-K<sup>b</sup> to ensure translocation into microsomes.

Translation products associated with microsomes were immunoprecipitated and analyzed by SDS-PAGE (Fig. 1, b and c). Translation of full-length Ii chain mRNA resulted in two products which were recognized equally well by mAb Bü45 (27) directed against the COOH terminus, and by mAb Vic-Y1, directed against the NH<sub>2</sub> terminus of Ii chain (26) (Fig. 1 b). The difference between these two polypeptides is not due to heterogeneity of glycosylation, since removal of N-linked carbohydrate chains with endo H still resulted in two polypeptides (Fig. 1 b). The COOH-terminally truncated Ii chains gave rise to two precursor proteins per mRNA as well, both of which were recognized by Vic-Y1 (Fig. 1 b). Translation of NH<sub>2</sub>-terminally truncated Ii chains resulted in only one translation product (Fig. 1 c) per mRNA. The difference between the two full-length Ii chain polypeptides therefore resides at the NH<sub>2</sub> terminus, most likely as the result of alternative initiation of translation. In view of the positions of methionines throughout the Ii chain and the sizes of the two Ii chain translation products, alternative initiation at position 15 is most probable (6). Initiation at the next methionine, at position 60, would have resulted in the absence of the cytoplasmic tail and therefore in the absence of the Vic-Y1 epitope.

The mAb Bü45, directed against the Ii chain luminal portion, recognized 183C and to some extent 163C, but not 153C (Fig. 1 b). The region COOH-terminal of amino acid 163 must therefore be important for the generation of the epitope recognized by this antibody. (Translation of 163C in this experiment produced the full-length Ii chain in addition to the truncated Ii chain. This is the result of incomplete digestion of the Ii chain cDNA with restriction enzyme *DrdI* before in vitro transcription, which was used to delete the COOH-terminal encoding part of the cDNA [see Materials and Methods].) The NH<sub>2</sub>-terminal deletion constructs were efficiently translated and translocated into microsomes. This was followed by cleavage of the H2-K<sup>b</sup> signal sequence (data not shown) and N-linked glycosylation of N81 and N110 as expected (Fig. 1 c). However, immunoprecipitation with Bü45, against the COOH terminus, was very inefficient (Fig. 1 c). The loss of the Bü45 epitope suggests that absence of the transmembrane region and cytoplasmic tail leads to misfolding of the remainder of the protein.

cysteine in the cytoplasmic tail. Migration of marker proteins are shown on the right. (c) mRNAs encoding NH<sub>2</sub>-terminally truncated Ii chains were translated in vitro. Microsome-associated products were divided into two parts and either directly loaded on gel (-) or immunoprecipitated with Bü45 (B). Samples were either mock treated (-) or digested with endo H (+).

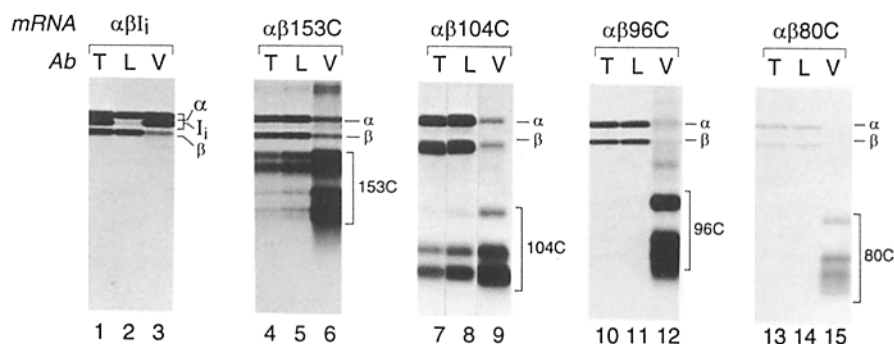
Glycosylation efficiency was reduced for COOH-terminal truncations shorter than 183 amino acids, leading to generation of two backbone and two glycosylated polypeptides (Fig. 1 *b*). The use of consensus glycosylation acceptor sequences (Asn-X-Thr/Ser) is thought to be dependent on the context in which they occur. Deletion of COOH-terminal sequences apparently leads to reduced accessibility of the glycosylation acceptor sites. For 104C and 96C (and 80C, Fig. 2) an endo H-sensitive polypeptide was observed in addition to two products of the expected size, although these constructs do not contain glycosylation acceptor sites. The occurrence of this aberrant polypeptide is not due to glycosylation of one of the properly sized chains, as removal of carbohydrates with endo H showed the appearance of a polypeptide of distinct mobility. The aberrant products were reactive with Vic-Y1, indicating that translation initiation was correct and had generated a cytoplasmic tail of the proper sequence. The possibility of a mutation in the cDNAs was excluded by DNA sequence analysis, which gave the expected primary structure. The synthesis of this glycosylated polypeptide can neither be explained by a shift in reading frame during translation, since the alternative two reading frames also do not contain glycosylation sites. We have not determined the exact location of attachment of this endo H-sensitive glycan, and have not found a satisfactory explanation for this phenomenon so far.

The COOH-terminally truncated constructs otherwise behaved in accordance with expectations as far as size, glycosylation, and reactivity with mAbs is concerned.

*Amino Acids 96–104, Located in the CLIP Region, Are Required for Association with Class II  $\alpha/\beta$  Heterodimers.* The ability of truncated Ii chains to associate with class II molecules was investigated by in vitro translation of these constructs together with class II HLA-DR1  $\alpha$  and  $\beta$  subunits in the presence of microsomes. After translation, microsomes were sedimented, lysed in detergent, and the extracts were subjected to immunoprecipitation. Interaction between Ii chain truncations and class II molecules was detected by immunoprecipitation with the following antibodies: mAb Tü36, which recognized both  $\alpha/\beta$  heterodimers and  $\alpha/\beta$ -Ii heterotrimers; mAb LB3.1, which recognizes  $\alpha/\beta$  heterodimers but not  $\alpha/\beta$ -Ii heterotrimers; and mAb Vic-Y1, which recognizes both free Ii chain and Ii chain associated with class II mole-

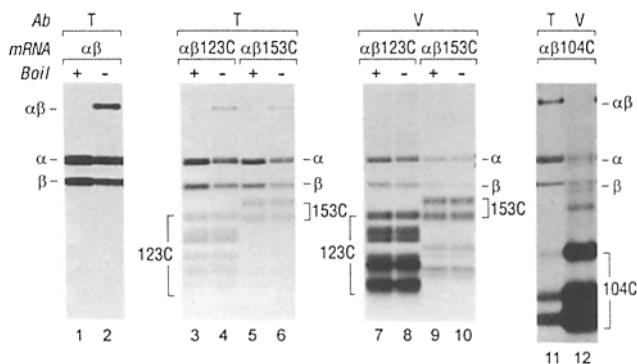
cules. All truncated Ii chains that extend beyond position 104 at the COOH terminus, were recovered with Tü36 and thus interact sufficiently strongly with class II molecules to survive immunoprecipitations (Fig. 2). In contrast to the full-length Ii chain, the COOH-terminally truncated Ii chains were also recovered with LB3.1 (Fig. 2). In metabolically labeled cells, this antibody recognizes  $\alpha/\beta$  heterodimers only after dissociation of the Ii chain. The COOH-terminal region of the Ii chain is thus responsible for obscuring the LB3.1 epitope on class II molecules. Immunoprecipitation of 104C and 153C with Vic-Y1 likewise showed association with  $\alpha$  and  $\beta$  chains (Fig. 2). Coimmunoprecipitation of  $\alpha$  and  $\beta$  chains with Vic-Y1 was completely inhibited in the presence of a synthetic Ii chain-derived peptide (residue 1–27) that is recognized by Vic-Y1 (data not shown). Note that predominantly the correctly glycosylated 153C polypeptides associated with class II molecules, whereas the unidentified aberrantly glycosylated 104C polypeptide did not (Fig. 2). Constructs 96C and 80C showed no trace of association with class II molecules, based on immunoprecipitation with Tü36 and LB3.1 (Fig. 2). The amount of  $\alpha$  and  $\beta$  chains recovered with Vic-Y1 from the translation with 104C was similar to that detected in the absence of Ii chain construct (data not shown), and can therefore be considered background level. Thus, the short region between amino acids 96 and 104, which constitutes the eight COOH-terminal amino acids of the CLIP region, is essential for association of Ii chains with class II molecules. Deletion of the luminal domain of Ii chain, beyond what is minimally required for interaction with class II molecules, has no effect on the formation of stable complexes. The NH<sub>2</sub>-terminal truncation N81, which contains the region essential for association, did not associate with class II molecules (data not shown). This confirms our notion that soluble forms of Ii chain produced in this manner are folded incorrectly.

*Association of Truncated Ii Chains with  $\alpha/\beta$  Heterodimers Correlates with Inhibition of Peptide Binding.* The presence of Ii chain precludes peptide binding to purified class II molecules (13). Binding of peptide can be monitored by the generation of SDS-stable  $\alpha/\beta$  dimers. These dimers do not dissociate upon incubation with SDS at room temperature, and can thus be visualized by SDS-PAGE. Boiling of the samples leads to full



**Figure 2.** Amino acids between 96 and 104 are essential for association with class II  $\alpha/\beta$  heterodimers. Full-length or COOH-terminally truncated Ii chains were translated in vitro together with class II HLA-DR1  $\alpha$  and  $\beta$  chains. Detergent lysed microsomes of these translations were divided into three equal parts and immunoprecipitated with mAbs Tü36 (T), which recognizes class II molecules regardless of the presence of Ii chain, LB3.1 (L), which recognizes free  $\alpha/\beta$  complexes but not (full-length) Ii chain-associated  $\alpha/\beta$  complexes, and Vic-Y1 (V), against the cytoplasmic tail of Ii chain. Immunoprecipitates of  $\alpha/\beta$ 104C were analyzed on a 10% polyacrylamide gel,

whereas the other immunoprecipitates were analyzed on 12.5% gels. These conditions are responsible for the difference in migration of  $\alpha$  and  $\beta$  chains between this translation and the others and the apparent faster migration of 104C compared with 96C.

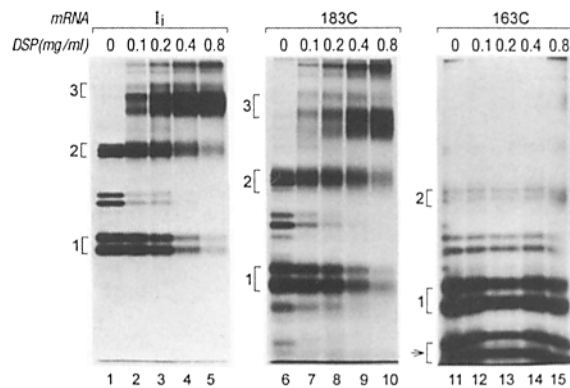


**Figure 3.** Association with COOH-terminally truncated Ii chains prevents peptide binding by class II molecules. Class II HLA-DR1  $\alpha$  and  $\beta$  chains were translated either alone or in the presence of COOH-terminal truncations of the Ii chain (123C, 153C, and 104C). After translation, detergent lysed microsomes were incubated with HLA-DR1 presentable peptide HA (306–308) (10  $\mu$ m) for 16 h on ice. Subsequently, extracts were divided into two equal portions and subjected to immunoprecipitations with either mAb Tü36 (T), specific for class II complexes, or mAb Vic-Y1 (V), specific for Ii chain. One half of the Staph.A-bound complexes were extracted at room temperature in SDS-sample buffer (Boil: –), to allow detection of SDS-stable  $\alpha/\beta$  dimers, the other half was extracted at 95°C (boil: +). The position of the SDS-stable  $\alpha/\beta$  dimers ( $\alpha/\beta$ ), as well as that of free  $\alpha$  and  $\beta$  chains and Ii chain truncations are indicated.

dissociation of these complexes into  $\alpha$  and  $\beta$  chains. Translation of  $\alpha$  and  $\beta$  chains, followed by incubation with the HLA-DR1 presentable peptide from influenza HA (306–318), led to recovery of SDS-stable dimers (Fig. 3). Translation of  $\alpha$  and  $\beta$  chains together with Ii chains leads to a mixture of  $\alpha/\beta$  heterodimers and  $\alpha/\beta$ -Ii heterotrimers, of which only the  $\alpha/\beta$  heterodimers are able to bind peptides, consistent with inhibition of peptide binding by the presence of Ii chain (29a).

To analyze which part of the Ii chain is responsible for inhibition of peptide binding, we investigated the ability of COOH-terminal truncations to carry out this function. Translation of either 104C, 123C, or 153C together with  $\alpha$  and  $\beta$  chains was followed by incubation with HA (306–318). This resulted in formation of complexes that were detectable with mAb Vic-Y1, against the Ii chain and Tü36, against  $\alpha/\beta$  complexes (Fig. 3). However, SDS-stable  $\alpha/\beta$  dimers were recovered with mAb Tü36 only (Fig. 3, lanes 4, 6, and 11), and not with Vic-Y1 (Fig. 3, lanes 7–10, 12). This indicates that  $\alpha/\beta$  heterodimers associated with Ii chain truncations do not contain peptide. Thus, interaction of Ii chain truncations with  $\alpha/\beta$  heterodimers leads to inhibition peptide binding.

*The Ii Chain Region Between Amino Acids 163 and 183 Is Involved in the Formation of Ii Chain Homotrimers.* In living cells, Ii chains assemble into homotrimers soon after synthesis (3). Trimerization could be demonstrated for Ii chains translated in vitro as well, when the homobifunctional cleavable cross-linker DSP was added shortly after lysis of microsomes. Increasing concentrations of DSP led to increasing amounts of trimers, and concomitantly decreasing amounts of monomers and dimers (Fig. 4). The Ii chain homodimers were detected independently of the presence of cross-linker under these



**Figure 4.** The region between amino acids 163 and 183 is important for visualization of homotrimerization by cross-linking. mRNAs of full-length or COOH-terminally truncated Ii chains were translated. After translation, microsomes were pelleted and lysed in 1 ml 1% NP-40 in PBS. The lysed microsomes were divided into five equal parts and the cross-linker DSP was added at the indicated concentrations. Quenching of cross-linker with glycine and removal of aggregates were done as described in Materials and Methods. Vic-Y1 immunoprecipitates were analyzed on 10% SDS-poly-acrylamide gel under nonreducing conditions. The positions of the monomers (1), dimers (2), and trimers (3) are indicated. The arrow indicates the nonglycosylated form of 163C.

nonreducing electrophoresis conditions. Their generation is most likely due to interchain disulfide bridge formation via the single cysteine located in the cytoplasmic tail of the Ii chain. Truncation 183C (which lacks the last 33 amino acids) showed the same oligomerization pattern as the full-length Ii chain (Fig. 4). However, 163C formed no homotrimers whatsoever, whereas homodimers were detected independently of the presence of DSP (Fig. 4). The stretch of amino acids between position 163 and 183 is therefore essential for the detection of homotrimers with DSP. Lysines, the  $\epsilon$ -NH<sub>2</sub> groups of which are the main target for cross-linking by DSP, are absent from this region. This leads us to conclude that the cross-linking occurs at a site NH<sub>2</sub>-terminal of residue 163.

## Discussion

We have located two distinct functional regions in the luminal part of the Ii chain by using in vitro translation of truncated Ii chains. The first segment is involved in formation of cross-linkable Ii chain homotrimers and is located between amino acids 163 and 183. This segment is likely to contribute to the architecture of the Ii chain trimer. Ii chain lacking this segment might still form trimers, which can no longer be cross-linked covalently. The second segment is involved in association of Ii chain with class II  $\alpha/\beta$  heterodimers and located closer to the membrane, between amino acids 96 and 104. We observed that replacement of the trans-membrane region and cytoplasmic tail by a cleavable signal sequence results in misfolding of the luminal part of Ii chains even though membrane insertion and glycosylation take place as expected.

Association with Ii chain determines the function of class II molecules in antigen presentation (8, 9). In contrast to class I molecules, which bind peptides from endogenously

synthesized proteins in the ER, class II molecules bind peptides from endocytosed proteins in endosomal compartments to which they are targeted by the Ii chain (15, 30–32). Interaction with Ii chain prevents peptide binding by class II molecules, but how this is executed and what region of the Ii chain is involved in this process is not understood. Two alternative mechanisms are plausible: (a) the Ii chain binds in the peptide binding groove and thus competes for antigenic peptide or (b) it binds outside the groove and prevents peptide binding by steric hindrance or by inducing a conformational change in the peptide binding domain.

Peptides derived from the Ii chain (CLIP peptides) have been eluted from several mouse and human class II molecules. These peptides originate from a region that is highly conserved between mouse, human, and rat (between amino acids 81 and 104 in mouse and human p33 Ii chain) (18). In *in vitro* peptide binding experiments, CLIP peptides efficiently compete with class II presentable peptides (21, 22) and incubation of empty class II molecules with CLIP peptides leads to generation of SDS-stable molecules (22, 29a). Thus, the CLIP peptides most likely bind in the peptide binding groove. Two possible processes could explain the binding of Ii chain-derived peptides: either the CLIP peptides are generated in the endocytic compartment by degradation of the dissociated Ii chain, or the CLIP region represents the region of Ii chain that interacts with class II molecules and remains associated after degradation of the Ii chain.

Freisewinkel et al. (18) have shown for the mouse Ii chain that the region between amino acids 87 and 110 (which corresponds to the same region in human p33 Ii chain) is essential for association with class II molecules. This result was obtained by transfecting COS cells with Ii chains carrying internal deletions rather than truncations. We have defined an even shorter region, between amino acids 96 and 104, with a similar function for the human Ii chain. Both the mouse and human segments essential for interaction with class II  $\alpha/\beta$  complexes, are located within the CLIP region (amino acids 81–104).

Freisewinkel et al. (18) pointed out that the membrane proximal location of the CLIP region does not agree with binding of the intact Ii chain via this region in the peptide binding groove of class II molecules. Indeed, only 24 amino acids separate the transmembrane region (residues 29–56) and the first

amino acid of the CLIP region (residue 81) (Fig. 1a). However, the “core” of the CLIP peptides (which are sets of peptides overlapping at COOH terminus as well as NH<sub>2</sub> terminus) is located 10 amino acids further away from the transmembrane region (at position 91). Chicz et al. (33) calculated that the length of the stretch of amino acids between the CLIP and transmembrane region, either in an extended conformation or even as an  $\alpha$  helix, may be sufficient to allow binding of the CLIP region in the peptide binding domain.

In our experiments, interaction of truncated Ii chains with class II molecules could not be separated from their ability to prevent peptide binding; even the shortest class II-associating construct (104 amino acids) prevented peptide binding. This indicates either that both functions are executed by the same region of the Ii chain or that the domain that inhibits peptide binding is located NH<sub>2</sub>-terminally of the domain essential for association with class II molecules. The COOH-terminal 102 amino acids do not seem to be not involved in either of these functions.

The issue of Ii chain–class II interaction and the mechanism by which Ii chain prevents peptide binding will only be definitively settled by elucidation of the three-dimensional structure of the Ii chain. For this purpose, it will be necessary to produce crystals of Ii chains derived from membrane anchored forms. Removal of the cytoplasmic tail and the transmembrane region leads to misfolding of the Ii chain in our *in vitro* translation system even though membrane insertion is successfully mediated by a cleavable NH<sub>2</sub>-terminal signal sequence. In intact Ii chain, folding of the luminal domain proceeds from a membrane tethered intermediate that apparently imposes constraints on the folding pathway. This aspect should be taken into account for successful folding of recombinant forms of Ii chain. Homotrimerization of the Ii chain might play a role in the acquisition of the proper conformation as well, and thereby be important for the assembly of  $\alpha$  and  $\beta$  chains. We have mapped a region involved in visualization of trimerization to amino acids 163–183. Interestingly, the stretch between amino acids 150 and 173 (LRHLKNTMETIDWKVFESWMHHL, underlined are the A and D positions) has the potential to fold into an amphiphatic helix with the possibility to form a coiled coil (34) which could constitute an obvious element to bring about trimerization.

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## References

1. Kvist, S., K. Wiman, L. Claesson, P.A. Peterson, and B. Dobberstein. 1982. Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell*. 29:61.
2. Machamer, C.E., and P. Cresswell. 1982. Biosynthesis and

- glycosylation of the invariant chain associated with HLA-DR antigens. *J. Immunol.* 129:2564.
3. Marks, M.S., J.S. Blum, and P. Cresswell. 1990. Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J. Cell Biol.* 111:839.
  4. Lamb, C., and P. Cresswell. 1992. Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. *J. Immunol.* 148:3478.
  5. Roche, P.A., M.S. Marks, and P. Cresswell. 1991. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature (Lond.)* 354:392.
  6. Claesson, L., D. Larhammer, L. Rask, and P.A. Peterson. 1983. cDNA clone for the human  $\gamma$  chain of class II histocompatibility antigens and its implications for the protein structure. *Proc. Natl. Acad. Sci. USA.* 80:7395.
  7. Strubin, M., C. Berte, and B. Mach. 1986. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3483.
  8. Cresswell, P. 1992. Chemistry and functional role of the invariant chain. *Curr. Opin. Immunol.* 4:87.
  9. Teyton, L., and P.A. Peterson. 1992. Invariant chain—a regulator of antigen presentation. *Trends Cell Biol.* 2:52.
  10. Schaiff, W.T., K.A. Hruska, Jr., D.W. McCourt, M. Green, and B.D. Schwartz. 1992. HLA-DR associates with specific stress proteins and is retained in the endoplasmic reticulum in invariant chain negative cells. *J. Exp. Med.* 176:657.
  11. Layet, C., and R.N. Germain. 1991. Invariant chain promotes egress of poorly expressed, haplotype-mismatched class II major histocompatibility complex  $A\alpha\beta$  dimers from the endoplasmic reticulum/cis-Golgi compartment. *Proc. Natl. Acad. Sci. USA.* 88:2346.
  12. Anderson, M.S., and J. Miller. 1992. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA.* 89:2282.
  13. Roche, P.A., and P. Cresswell. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature (Lond.)* 345:615.
  14. Teyton, L., D. O'Sullivan, P.W. Dickson, V. Lotteau, A. Sette, P. Fink, and P.A. Peterson. 1990. Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature (Lond.)* 348:39.
  15. Lotteau, V., L. Teyton, A. Peleraux, T. Nilsson, L. Karlsson, S.L. Schmid, V. Quaranta, and P.A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature (Lond.)* 348:600.
  16. Bakke, O., and B. Dobberstein. 1990. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell.* 63:707.
  17. Roche, P.A., and P. Cresswell. 1991. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc. Natl. Acad. Sci. USA.* 88:3150.
  18. Freisewinkel, I., K. Scenck, and N. Koch. 1993. The segment of invariant chain that is critical for association with major histocompatibility complex class II molecules contains the sequence of a peptide eluted from class II polypeptides. *Proc. Natl. Acad. Sci. USA.* 90:9703.
  19. Hunt, D.F., H. Michel, T.A. Dickinson, J. Shabanowitz, A.L. Cox, K. Sakaguchi, E. Appella, H.M. Grey, and A. Sette. 1992. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science (Wash. DC).* 256:1817.
  20. Rudensky, A.Y., P. Preston-Hurlburt, S.C. Hong, A. Barlow, and C.A. Janeway. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature (Lond.)* 353:622.
  21. Riberdy, J.M., J.R. Newcomb, M.J. Surman, J.A. Barbosa, and P. Cresswell. 1992. HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature (Lond.)* 360:474.
  22. Chicz, R.M., R.G. Urban, W.S. Lane, J.C. Gorga, L.J. Stern, D.A.A. Vignali, and J.L. Strominger. 1992. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogenous in size. *Nature (Lond.)* 358:764.
  23. Larhammer, D., K. Gustafsson, L. Claesson, P. Bill, K. Wiman, L. Schenning, J. Sundelin, E. Widmark, P.A. Peterson, and L. Rask. 1982. Alpha chain of HLA-DR transplantation antigens is a member of the same protein superfamily as the immunoglobulins. *Cell.* 30:153.
  24. Bell, J.I., P. Estess, T.S. John, R. Saiki, D.L. Watling, H.A. Erlich, and H.O. McDevitt. 1985. DNA sequence and characterization of human class II major histocompatibility complex  $\beta$  chains from the DR1 haplotype. *Proc. Natl. Acad. Sci. USA.* 82:3405.
  25. Shaw, S., A. Ziegler, and R. DeMars. 1985. *Hum. Immunol.* 12:191.
  26. Quaranta, V., O. Majdic, G. Stingl, K. Liszka, H. Honigsmann, and W. Knapp. 1984. A human Ia cytoplasmic determinant located on multiple forms of invariant chain ( $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ ). *J. Immunol.* 132:1900.
  27. Wraight, C.J., P. Van Endert, P. Möller, J. Lipp, N.R. Ling, I.C.M. MacLennan, N. Koch, and G. Moldenhauer. 1990. Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J. Biol. Chem.* 265:5787.
  28. Rothbard, J.B., R.I. Lechler, K. Howland, V. Bal, D.D. Eckels, R. Sékaly, E.O. Long, W.R. Taylor, and J.R. Lamb. 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. *Cell.* 52:515.
  29. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.
  - 29a. Bijlmakers, M.E., P. Benaroch, and H.L. Ploegh. 1994. Assembly of HLA-DR1 molecules translated *in vitro*: binding of peptide in the endoplasmic reticulum precludes association with invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.
  30. Cresswell, P. 1985. Intracellular class II HLA antigens are accessible to transferrin-neuraminidase conjugates internalized by receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 82:8188.
  31. Neefjes, J.J., V. Stollorz, P.J. Peters, H.J. Geuze, and H.L. Ploegh. 1990. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell.* 61:171.
  32. Peters, P.J., J.J. Neefjes, V. Oorschot, H.L. Ploegh, and H.J. Geuze. 1991. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature (Lond.)* 349:669.
  33. Chicz, R.M., R.G. Urban, J.C. Gorga, D.A.A. Vignali, W.S. Lane, and J.L. Strominger. 1993. Specificity and promiscuity among naturally processed peptide bound to HLA-DR alleles. *J. Exp. Med.* 178:27.
  34. Cresswell, P. 1994. Assembly, transport and function of MHC class II molecules. *Annu. Rev. Immunol.* 12:259.