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Immunology

Assembly and peptide binding of major histocompatibility complex class II heterodimers in an *in vitro* translation system

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ABSTRACT **_In vitro_ transcription/translation of HLA-DR1 cDNAs in the presence of microsomal membranes was used to study the association of major histocompatibility complex class II molecules with peptide and invariant chain (Ii) in the endoplasmic reticulum (ER). HLA-DRα and HLA-DRβ subunits assembled into SDS-unstable heterodimers in the absence of exogenous peptide. The inclusion of synthetic peptides during the α/β assembly process promoted their conversion to SDS-resistant heterodimers. Addition of Ii RNA during the translation of HLA-DRα and HLA-DRβ RNAs resulted in the formation of α/β/Ii complexes. Peptide binding by class II molecules was detected even when excess Ii was present during α/β assembly. These findings indicate that peptides can bind α/β heterodimers in the ER microenvironment and suggest that peptides derived from cytosolic proteins that are presented by class II molecules at the cell surface may have bound to HLA-DR in the ER.**

Major histocompatibility complex (MHC) class II HLA-DRα and HLA-DRβ chains assemble with each other and invariant chain (Ii) in the endoplasmic reticulum (ER). The physical association of Ii with MHC class II heterodimers is thought to block premature peptide binding, but whether this is an allosteric effect or involves steric obstruction of the peptide binding groove is not known (1–3). Once associated with Ii, MHC class II molecules are trafficked to endosomes by a targeting signal on the cytoplasmic tail of Ii (4, 5). While en route to the cell surface, Ii is proteolytically removed (6) and class II molecules then bind peptides before being transported to the cell surface.

Analysis of peptides extracted from human MHC class II molecules has identified a small percentage of HLA-DR-bound peptides that were derived from cytoplasmic source proteins (3, 7). In addition, other reports have suggested that class II molecules can bind peptides in the ER (8–12). While these data suggest that HLA-DR might bind peptide in a preendosomal compartment, the precise cellular location in which these peptides were bound has not been determined.

Upon binding most types of peptide, class II heterodimers become resistant to SDS-induced dimer cleavage and migrate as a 60-kDa complex on SDS/PAGE (13, 14). Interestingly, class II molecules expressed in Ii-deficient mice are not SDS stable (15–17), implying that these class II heterodimers are devoid of peptide. Recently, however, antigen presenting cells from Ii-deficient mice, which are also transgenic for epitopes from myelin basic protein, have been shown to present this endogenous antigen to T cells, thus demonstrating that some fraction of class II molecules in Ii^0 mice are peptide loaded and functional (18). Moreover, not all peptides induce the formation of SDS-stable class II molecules upon binding (19), and it is possible that the peptides available for association with class II in the ER may be of this type. Thus, data from studies on class II-restricted endogenous antigen presentation and the Ii-deficient mice have not resolved whether peptide–class II interaction can occur in the ER.

To definitively address this question, *in vitro* transcription/translation in the presence of canine pancreatic microsomal membranes was used to analyze the assembly of HLA-DRα, HLA-DRβ, and/or Ii molecules and to determine their peptide binding capabilities.

MATERIALS AND METHODS

Antibodies. DA6.147 and PIN1 were gifts from P. Cresswell (Yale University). Rabbit anti-DRβ1 antiserum was a gift from H. Ploegh (Massachusetts Institute of Technology). BU43 was a gift from I. C. M. MacLennan (Birmingham, U.K.).

Peptides. All peptides were synthesized by standard 9-fluorenylmethoxycarbonyl chemistry, purified by reversed-phase chromatography, and verified by mass spectrometry; the final concentrations were determined by quantitative amino acid analysis or weight.

Cloning. cDNAs for HLA-DRA1 and HLA-DRB1*0101 were cloned into pGEM2 (Promega) and pCITE (Novagen), respectively. The 5′ untranslated region of the HLA-DRβ cDNA was removed to improve translation efficiency. Ii cDNA was cloned into pSELECT-1 (Promega). The first ATG (Ii p35 translation start site) was mutated to ATC by site-directed mutagenesis as described by the manufacturer of the Altered Sites *in vitro* mutagenesis system (Promega).

***In Vitro* Transcription and Translation.** Plasmid DNAs were linearized with the appropriate restriction enzymes and RNA was transcribed using the ribomax T7 kit from Promega. Transcriptions were performed following the instructions provided by the manufacturer.

Translations were performed using the conventional or flexi-rabbit reticulocyte translation kits (Promega). The protocol provided by the manufacturer was followed with the addition of 2.5 μl of [^{35}S]methionine (Amersham) and 1.5 μl of canine pancreatic microsomal membranes. KCl (0.8 μl per 25 μl of reaction mixture) was added when the flexi-rabbit reticulocyte translation kit was used. When the conventional reticulocyte lysate kit was used, 1.5–2 mM oxidized glutathione was added to the translation mixtures. Translations were for 60–90 min at 37°C. Samples were spun at 14,000 × *g* for 5 min to pellet the membranes and then washed in 0.5 ml of 130 mM KCl/66 mM Tris·HCl, pH 7.5/33 mM EDTA, pH 8.0, and repelleted for 10 min. The membranes were solubilized in 40 μl of 150 mM NaCl/50 mM Tris·HCl, pH 7.5/1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride/10 mM aprotinin/10 mM leupeptin/10 mM iodoacetamide.

Immunoprecipitations. For immunoprecipitations, 35 μl of membranes was mixed with 270 μl of immunoprecipitation

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Abbreviations: MHC, major histocompatibility complex; Ii, invariant chain; ER, endoplasmic reticulum; HA, hemagglutinin; ApoB, apolipoprotein B.

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(IP) buffer containing 225 mM NaCl, 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40. Purified antibody (1 mg/ml) was added to achieve a dilution of 1:500. The samples were incubated at room temperature (RT) for 1–2 hr. Protein A-Trisacryl (15 μl) and protein G-Sepharose (15 μl) 50% slurries (both preincubated in 1% bovine serum albumin for 15 min and washed in IP buffer before use) were added to the samples and incubated for 1–2 hr. Samples were washed in IP buffer four times and the beads were resuspended in 1% SDS sample loading buffer. Samples were boiled or incubated at RT for 10–60 min before being loaded onto SDS/12% polyacrylamide gels. After electrophoresis, the gels were fixed and dried. Densitometric analysis was performed on a Fuji BAS2000 bioimager.

RESULTS

***In Vitro* Translation Assay and Antibody Specificity.** cDNAs encoding Ii, HLA-DRA1, and HLA-DRB1*0101 proteins were cloned into expression vectors containing a T7 RNA polymerase promoter. To distinguish between HLA-DRα and Ii proteins by one-dimensional SDS/PAGE, the p35 alternative translation start site in the Ii cDNA was mutated so that only the p33 form of the protein was produced (see *Materials and Methods*). *In vitro* translation of the RNAs encoding HLA-DRα, HLA-DRβ, and Ii generated proteins that, based on their migration in SDS/polyacrylamide gels, were appropriately modified by signal sequence cleavage and N-linked glycosylation (data not shown).

To verify the specificity of the antibodies used in the immunoprecipitation experiments, HLA-DRα, HLA-DRβ, and Ii RNAs were translated and the resulting proteins were immunoprecipitated with a panel of antibodies. The data show that only DA6.147 precipitated HLA-DRα (Fig. 1, lanes 1–4), a rabbit antiserum developed against gel-purified HLA-DRβ chains was the only reagent to react with the HLA-DRβ chain (lanes 5–8), and PIN1 was the only antibody to recognize Ii (lanes 9–12). LB3.1, a HLA-DRα/β complex-dependent monoclonal antibody, did not recognize any of the single chains (lanes 2, 6, and 10).

Assembly of the HLA-DR Heterodimer. *In vitro* transcription/translation has been successfully used to study MHC class I assembly (20–22). In this report, a similar system was used to study α/β and α/β/Ii assembly.

HLA-DRα and HLA-DRβ RNAs were cotranslated or translated independently and the products were immunoprecipitated with the HLA-DRα-specific monoclonal antibody DA6.147 (Fig. 2). Since HLA-DRβ is coimmunoprecipitated when both chains are translated and immunoprecipitation with LB3.1, a conformation-dependent antibody, also recognized the *in vitro* translated HLA-DR1 heterodimer, suggesting that it is folded correctly (data not shown), these data clearly demonstrate that the HLA-DR1 heterodimer forms in the absence of exogenous peptide or Ii and indicate that the *in vitro* translated products assemble.

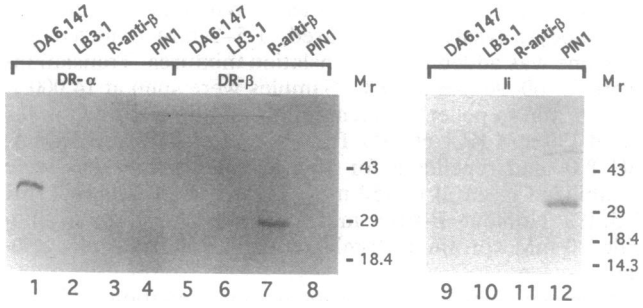

FIG. 1. Immunoprecipitation of HLA-DRα, HLA-DRβ, and Ii from *in vitro* translations. After immunoprecipitation, the products were resuspended in SDS loading buffer and analyzed by SDS/PAGE. Lanes: 1–4, HLA-DRα; 5–8, HLA-DRβ; 9–12, Ii and M_r markers (×10^{-3}). Antibodies used in each immunoprecipitation are indicated above the lane. R-anti-β, rabbit HLA-DRβ antiserum.

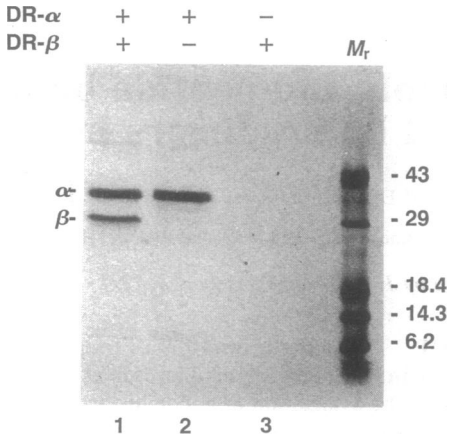

FIG. 2. *In vitro* formation of HLA-DRα and HLA-DRβ heterodimers. HLA-DRα and HLA-DRβ RNAs were cotranslated (lane 1) or translated independently (lanes 2 and 3) and immunoprecipitated with DA6.147. Lane M_r, molecular weight markers (×10^{-3}).

Association of Ii with HLA-DRα and HLA-DRβ. It has been reported that Ii can associate with individual HLA-DR chains (2, 5, 23) and with assembled α/β heterodimers (24). The translation/transcription system was used to determine whether these interactions are detectable in the *in vitro* translation system. In each of these experiments the RNAs were titrated to achieve equimolar amounts of HLA-DRα and HLA-DRβ chains and a molar excess of Ii. The intensity of the immunoprecipitated bands is related to the number of methionines in each of the three proteins. The DA6.147 monoclonal antibody immunoprecipitated HLA-DRα from the α/Ii cotranslation, but Ii was not coimmunoprecipitated (Fig. 3, lane 6). PIN1 and BU43 immunoprecipitated Ii from the α/Ii mixture but did not immunoprecipitate detectable

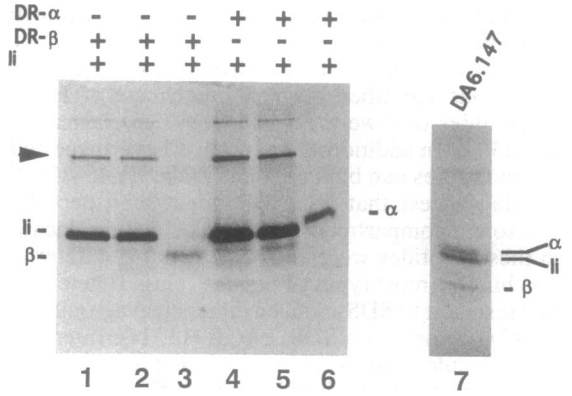

FIG. 3. Ii does not associate with HLA-DRα or HLA-DRβ chains alone but does associate with the HLA-DRα/β heterodimer. HLA-DRβ and Ii RNAs were cotranslated (lanes 1–3) and immunoprecipitated with BU43 (lane 1), PIN1 (lane 2), or rabbit HLA-DRβ antiserum (lane 3). HLA-DRα and Ii RNAs were cotranslated (lanes 4–6) and immunoprecipitated with BU43 (lane 4), PIN1 (lane 5), or DA6.147 (lane 6). HLA-DRα, HLA-DRβ, and Ii RNAs were cotranslated (lane 7) and immunoprecipitated with DA6.147. Note that the number of methionine residues in each protein is different (Ii, 15; HLA-DRα, 3; HLA-DRβ, 2), so that autoradiography of equimolar amounts of protein would reveal a band representing Ii that is stronger than the bands representing HLA-DRα or HLA-DRβ. Arrowhead indicates a putative Ii homodimer. A small amount of a slower-migrating band that may represent an Ii homotrimer is also evident.

levels of HLA-DRα (lanes 4 and 5). Both PIN1 and BU43, which recognize different domains of Ii, were used in these experiments in the event that single-chain–Ii interactions might interfere with antibody recognition. Similar results were seen when the β/Ii cotranslation was analyzed. Rabbit HLA-DRβ antiserum immunoprecipitated the HLA-DRβ chain but not Ii chain (lane 3), and the Ii-specific antibodies immunoprecipitated only Ii from the β/Ii mixture (lanes 1 and 2). Although Ii does not appear to bind single HLA-DR subunits in these experiments, the possibility that these interactions occur at levels not detectable by this assay or that the antibodies used here do not react with α/Ii or β/Ii complexes is not excluded.

The lack of detectable single-chain interactions with Ii made it feasible to use these antibodies to examine α/β/Ii assembly. When HLA-DRα, HLA-DRβ, and Ii RNAs were cotranslated and the products were analyzed by immunoprecipitation, all three proteins were coimmunoprecipitated with DA6.147 (Fig. 3, lane 7), demonstrating that α/β/Ii complexes were generated in the microsomes.

Peptide Binding to HLA-DR1. Since synthetic peptides are able to enter microsomes (20, 25, 26), and, as shown above, HLA-DR1 molecules assemble in these vesicles, experiments were done to determine whether peptide binding could occur in this microenvironment. The binding of most peptides induces stability of MHC class II heterodimers in SDS/polyacrylamide gels (13, 14). Therefore, the following assay was used to monitor HLA-DR–peptide interaction. HLA-DRα and HLA-DRβ RNAs were cotranslated in the presence of increasing amounts of an A2-like peptide (Table 1, ref. 27). The membranes were pelleted, washed extensively to remove excess peptide (peptide was diluted at least 1:1000 after the washes), solubilized in 1% Nonidet P-40, and immunoprecipitated with DA6.147. The samples were separated on SDS/polyacrylamide gels without prior boiling. In the absence of added peptide, all of the assembled HLA-DR molecules were SDS unstable; however, as the concentration of the synthetic peptide was increased (10–400 μM), a linear increase in the number of SDS-resistant molecules was observed (Fig. 4*A*).

With this assay, other HLA-DR1 binding peptides (Table 1) were tested for their ability to induce SDS stability. Several synthetic peptides conferred SDS stability to HLA-DR1 heterodimers, including fetuin, IgKappa (κ), hemagglutinin (HA), and apolipoprotein B (ApoB) peptides (Fig. 4*B*), indicating that the ability to bind HLA-DR1 in the ER microenvironment is not limited to one peptide.

Some synthetic peptides such as Ii15 and Ii24 and the HLA-DRα peptide that have previously been found associated with HLA-DR1 did not induce detectable levels of SDS-stable HLA-DR1 heterodimers when present during translation (Fig. 4*B*, lane 5; data not shown). However, when HLA-DRα and HLA-DRβ were cotranslated, the microsomes were solubilized, and peptide was then added to the reaction mixtures, the Ii15 and Ii24 peptides were able to promote the formation of SDS-stable HLA-DR molecules (Fig. 4*C*, lanes 3 and 4). Ii24 was previously shown to induce

Table 1. Peptides tested for binding to HLA-DR1

Peptide	Amino acid sequence
Ii15	KMRMATPLLMQALPM
Ii24	LPKPPKPVSKMRMATPLLMQALPM
κ	KHKVYACEVTHQGLS
DRα	APSPLPETTENVVCALGL
ApoB	IPDNLFLKSDGRIKYTLNK
HA	PKYVKQNTLKLAT
Fetuin	YKHTLNQIDSVKVWPRRP
A2-like	VGSDWRFLRGYHQYAYDG

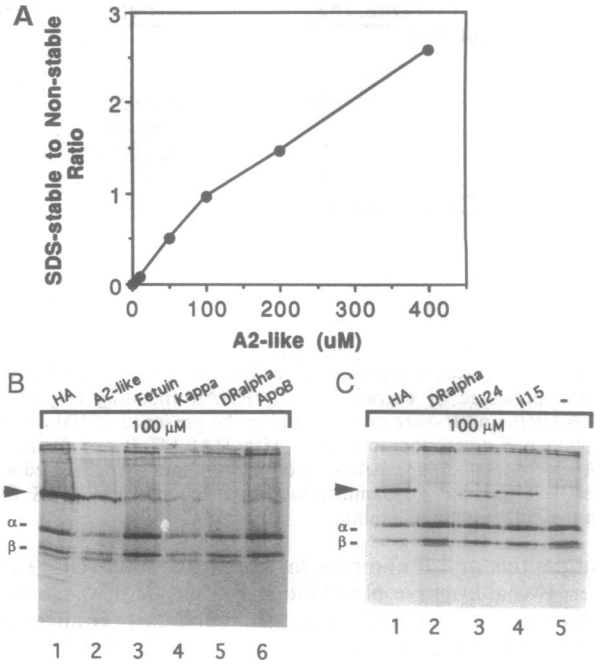

FIG. 4. Formation of SDS-stable HLA-DR heterodimers by addition of synthetic peptides. (*A*) HLA-DRα and HLA-DRβ RNAs were cotranslated in the absence or presence of A2-like peptide. Concentration of peptide in each reaction mixture is indicated on the *x* axis. The ratio of SDS-stable to -unstable heterodimers formed in each translation (*y* axis) was quantified on a Fuji BAS2000 bioimager after immunoprecipitation with DA6.147. (*B*) *In vitro* translation of HLA-DRα and HLA-DRβ RNAs was performed in the presence of 100 μM peptide. Peptide added to each translation mixture is indicated above each lane. Samples were immunoprecipitated with DA6.147 and analyzed by SDS/PAGE without prior boiling. SDS-stable heterodimer is indicated by arrowhead. (*C*) After cotranslation of HLA-DRα and HLA-DRβ, microsomes were solubilized and peptide was added at a concentration of 100 μM. Samples were incubated at 37°C for 60 min, immunoprecipitated with DA6.147, and loaded onto gels in 1% SDS sample buffer without boiling. Peptide added to each sample is indicated above the lanes; −, no peptide was added to the translation mixture. SDS-resistant form of HLA-DR is indicated by an arrowhead.

SDS stability of HLA-DR1 (27) but does not induce SDS stability of HLA-DR3 (28–30), and these differences are likely to be allotype specific. The HLA-DRα peptide did not induce SDS-stable molecules even when incubated with HLA-DR after solubilization of the membranes (Fig. 4*C*, lane 2), indicating that, although this peptide may bind HLA-DR molecules, it does not induce SDS stability in this assay. The integrity of this peptide was subsequently confirmed by HPLC analysis (data not shown).

The Effect of Ii on Peptide Binding. The data presented here demonstrate that peptides interact with HLA-DR in an ER-like microenvironment. Whether this occurs *in vivo* is likely to depend on the ability of a peptide to bind HLA-DR in the presence of Ii. The *in vitro* translation/assembly assay was used to determine whether peptides could bind HLA-DR1 in the presence of Ii. HLA-DRα, HLA-DRβ, and Ii RNAs were cotranslated in the presence or absence of 100 μM HA peptide. The interaction of class II molecules with either peptide or Ii was then assayed by immunoprecipitation. Both SDS-stable molecules (HLA-DR/peptide complexes) and α/β/Ii complexes were detected when HA was included in the translation mixture (Fig. 5, lane 2). Therefore, when Ii RNA is cotranslated with the HLA-DRα and HLA-DRβ RNAs, the HA peptide can bind HLA-DR and induce SDS-stable heterodimers (although the α/β/Ii complexes are SDS-unstable under these conditions). These data suggest

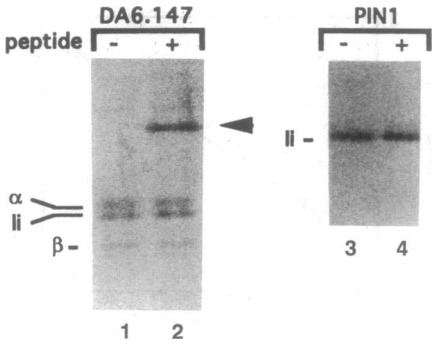

FIG. 5. Synthetic peptides induce SDS-stable HLA-DR1 dimers in the presence of cotranslated Ii. RNAs encoding HLA-DRα, HLA-DRβ, and Ii were cotranslated in the presence of 100 μM HA peptide and immunoprecipitated with DA6.147 (lanes 1 and 2). DA6.147-depleted supernatants were then immunoprecipitated with PIN1 (lanes 3 and 4). Samples were loaded onto gels in 1% SDS sample buffer without boiling.

that peptide and Ii compete for binding to HLA-DR. However, two alternative possibilities are that (*i*) Ii was limiting in the microsomes, and the non-Ii-bound class II molecules were free to interact with peptide; and (*ii*) HLA-DR1 molecules that for some reason were unable to interact with Ii were able to bind peptide. To address these possibilities the following experiments were performed.

To determine whether Ii was in excess, sequential immunoprecipitations were performed on the samples in Fig. 5. After removal of the α/β/peptide and α/β/Ii complexes with DA6.147, the samples were immunoprecipitated with PIN1 to measure the amount of excess Ii. It is clear from these experiments that free Ii is present in the translation mixtures (Fig. 5, lanes 3 and 4).

To ascertain whether two distinct populations of HLA-DR molecules were present—one that was able to interact with Ii and one that was able to bind peptide—the following experiment was done. Increasing amounts of Ii RNA were added to translation mixtures containing the A2-like or HA synthetic peptides and HLA-DRα and HLA-DRβ RNAs. As the level of Ii RNA was increased to saturating levels, the ratio of SDS-stable to non-SDS-stable heterodimers decreased (Fig. 6); however, HLA-DR1 molecules bound to peptide were still detectable. These results demonstrate that the

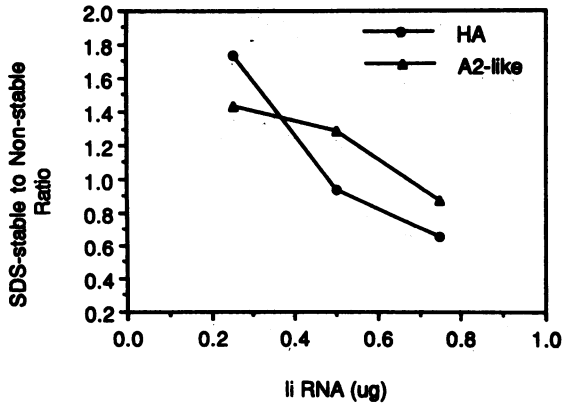

FIG. 6. Ii RNA concentration affects the level of peptide-induced SDS-stable heterodimer formation. HA (100 μM) or A2-like synthetic peptide was added during each α/β/Ii cotranslation. Amounts of HLA-DRα and HLA-DRβ RNAs were held constant but the Ii RNA concentration (*x* axis) was increased until HLA-DRα and HLA-DRβ translation was markedly decreased, thereby ensuring a vast excess of Ii in the microsomes. Ratio of SDS-stable to -unstable heterodimers formed in each translation mixture was quantified on a Fuji BAS2000 bioimager after immunoprecipitation (*y* axis).

population of HLA-DR molecules that bind peptide in the ER is likely to be the same as that which associates with Ii and suggest that, although peptides can bind HLA-DR in the presence of Ii, most of the heterodimers are associated with Ii.

DISCUSSION

The studies presented here describe an *in vitro* system designed to study peptide binding and assembly of MHC class II heterodimers. In this system, HLA-DRα, HLA-DRβ, and Ii RNAs are cotranslated in the presence of microsomal membranes that simulate the microenvironment of the ER. α/β complexes assemble in this system and bind peptides as assayed by stable heterodimer formation in SDS/PAGE. α/β/Ii complexes also assemble and synthetic peptides are able to bind HLA-DR in the presence of Ii.

Ii is thought to prevent class II proteins from binding peptide in the ER (1–3). However, most class II molecules of Ii-deficient mice are devoid of SDS-stabilizing peptides (15–17), suggesting that aside from Ii there are other reasons for the apparent inability of these class II molecules to bind peptides in the ER. These may include the following:

(*i*) The environment in the ER is not suitable for peptide binding. In fact, at least one *in vitro* study indicated that the k_{on} rate for peptide binding is enhanced at acidic pH (31). Although the low pH in the normal class II peptide loading compartments may promote class II–peptide interactions, the results presented here demonstrate that there is no physiochemical obstacle for peptide binding in the ER.

(*ii*) The requirement for a specialized peptide loading system may preclude the class II–peptide interaction in the ER. There are several hypotheses that invoke the existence of a mechanism to load peptides onto class I proteins in the ER (20, 32–35), but the efficiency with which class II molecules might be loaded by this putative system is unclear. Since peptide–class II interaction normally occurs in specialized vesicles (36–38) and not the ER, this compartment is unlikely to have the machinery required to promote class II peptide loading.

(*iii*) Calnexin and other stress-related proteins that associate with HLA-DR in the ER could retain class II molecules until assembly of the heterodimer or association with Ii occurs (39–41). Association with calnexin or other chaperones may also interfere with class II–peptide interactions.

(*iv*) MHC class I binding peptides are transported into the ER lumen primarily through the TAP transporter (26, 42). It is unclear whether the synthetic peptides used here entered the microsomes via TAP. Some studies have determined that the C-terminal residue of a peptide has an effect on its TAP-dependent transport efficiencies, but no simple motif or upper size limit has been defined that consistently makes certain peptides better substrates than others (26, 42–45). Moreover, peptides are able to gain access to the ER in the absence of TAP transporters (20, 46). For these reasons, it is not possible to predict which of the peptides used in these studies would be the most efficient at membrane translocation.

Membrane pumps that remove peptides from the ER (44), or the presence of chaperones such as GP96 and BiP which bind peptides rich in hydrophobic residues (35, 47), may decrease the effective concentration of these peptides within the ER. Given the slow rate at which HLA-DR binds peptide, the interaction would not be favored under conditions of limiting peptide. The importance of peptide concentration is underscored by results which show that higher concentrations of peptide promote an increase in the number of HLA-DR molecules that acquire SDS resistance (i.e. Fig. 4).

For the reasons listed above, there may be restraints on the type of peptide able to bind HLA-DR in the ER, but it is clear

that *in vitro* class II molecules can interact with peptides in this microenvironment. An important concern is the frequency with which this occurs *in vivo*. There are examples of HLA-DR binding to peptides derived from cytosolic proteins (3, 7, 11, 48). Given the data presented here, one interpretation for these results is that class II molecules may have acquired these cytosolic peptides in the ER. Several criteria will determine the efficiency of this process *in vivo*, the most important of these being whether a given peptide can achieve a concentration within the ER that enables it to effectively compete with Ii (10).

In addition to demonstrating that peptide binds HLA-DR in the microenvironment of the ER, this report describes an *in vitro* assay that will be a valuable tool in the analysis of peptide-facilitated class II assembly and in characterizing mutations that may affect the assembly process.

Note added in proof: A report (49) describing similar results has recently appeared.

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