The MHC is central to the adaptive immune response. The human MHC class II is encoded by three different isotypes, HLA-DR, -DQ and -DP, each being highly polymorphic. In contrast to HLA-DR, the intracellular assembly and trafficking of HLA-DP molecules has not been extensively studied. However, different HLA-DP variants can be either protective or risk factors for infectious diseases (e.g. Hepatitis B), immune dysfunction (e.g. berylliosis) and autoimmunity (e.g. myasthenia gravis). Here, we establish a system to analyze the chaperone requirements for HLA-DP and to compare the assembly and trafficking of HLA-DP, -DQ and -DR directly. Unlike HLA-DR1, HLA-DQ5 and HLA-DP4 can form SDS-stable dimers supported by invariant chain (Ii) in the absence of HLA-DM. Uniquely, HLA-DP also forms dimers in the presence of HLA-DM alone. In model antigen presenting cells, SDS-stable HLA-DP complexes are resistant to treatments that prevent formation of SDS-stable HLA-DR complexes. The unexpected properties of HLA-DP molecules may help explain why they bind to a more restricted range of peptides than other human MHC class II proteins and frequently present viral peptides.

INTRODUCTION

MHC class II molecules play an important role in the immune system. They are essential in the defense against infection and are a main consideration in transplantation medicine. In addition to presenting antigenic peptides from predominantly extracellular sources to CD4+ T cells, MHC class II molecules also mediate the thymic selection of helper T cells. MHC class II molecules consist of an α and β chain and are transported to endosomal/lysosomal compartments by the Ii. The Ii is degraded until only a small fragment, dubbed CLIP, remains bound in the peptide-binding groove. Lysosomal pH and the class II-like molecule HLA-DM promote the exchange of the CLIP fragment for more stably binding antigenic peptides (1).

In humans, MHC class II molecules are encoded by three different loci, HLA-DR, -DQ and -DP, that display ~70% similarity to each other. Polymorphism is a notable feature of MHC class II genes. For HLA-DR, most variability comes from DRB, with over 700 known alleles at population level, whereas there are only three DRA variants. In contrast, both chains of HLA-DQ and -DP are polymorphic (2). For HLA-DP, however, only a few alleles are prevalent, most notably the heterodimer DPA1*0103/DPB1*0401 (DP401) (3).

Despite the essential function of MHC class II molecules in immune defense against pathogens, some alleles are frequently linked to immune diseases. For example, HLA-DR1 and DR4 predispose for rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus, while DR2 confers susceptibility to multiple sclerosis. Similarly, DQ2 and DQ8 are linked to coeliac disease (4,5). The role of HLA-DR in immune dysfunction has been less well defined. However, DP0201 is a risk factor for the autoimmune disease myasthenia gravis in the Japanese (6) and DP alleles with a glutamic acid at position 69 are associated with berylliosis, a hard metal lung disease (7). Although presentation of intracellular antigens by MHC class II molecules is considered atypical, HLA-DP4 gene products frequently present viral peptides, for example from HIV envelope protein, rabies virus and Hepatitis B virus envelope protein (8,9).

DR1 (DRA, DRB1*0101) was the first MHC class II molecule to be crystallized (10), and HLA-DR is the most intensively studied MHC class II isotype. Efficient peptide presentation by HLA-DR is well-recognized to
depend on both Ii and DM. Indeed, biochemical studies suggested that HLA-DR alleles that bind inefficiently to the li CLIP fragment are more likely to induce an autoimmune response, for example in rheumatoid arthritis (11). Weak affinity of the li for DQ has also been associated with juvenile dermatomyositis (12). Structural information has been obtained for some HLA-DQ molecules involved in autoimmune disease; for example, crystal structures of the DQ8-insulin peptide complex (13) and the DQ2-gluten peptide complex have been solved (14). Although SDS-stable DQ molecules have been visualized (15), and li supports assembly of the DQ-like H-2A protein in the mouse (16), the relative contributions of DM and li in the acquisition of stable DQ\(\alpha \beta\) dimers has not been fully explored. The first crystal structure of an HLA-DP protein, HLA-DP2, has been recently published, in complex with a self-peptide from the DP\(\alpha\) chain (17). HLA-DP molecules bind a limited set of peptides (18), but the relative lack of molecular and biochemical studies on HLA-DP means that exactly how it acquires peptides is unclear.

The organization and expression of the MHC, particularly of DP-like genes, varies greatly between mammals, making comparative study of DP function in model animals difficult. In mice, which lack functional DP paralogues, I-E and I-A are considered the operative homologs of DR and DQ, respectively. Unlike HLA-DQ and DR, HLA-DPB1 sequences from humans, macaques and great apes group into distinct lineages, suggesting that DP evolution has occurred post-speciation (19).

To overcome the limitations of animal models with respect to HLA-DP biochemistry, we have employed a human cell culture system to directly compare the assembly and trafficking of DP with DQ and DR. Notably, in an identical cellular environment, DR, DQ and DP have different requirements for li and DM. Our results suggest that trafficking and peptide-loading of different MHC class II molecules can be modulated by tuning the level of DM and li in APCs, and our data have implications for the role of HLA-DP in autoimmune disease.

**EXPERIMENTAL PROCEDURES**

*Cell lines and antibodies*

Human cervical carcinoma HeLa cells and human melanoma MeJuso cells were maintained in MEM or DMEM (Invitrogen), respectively, supplemented with 8% fetal calf serum (Sigma), 2 mM glutamax, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin (Invitrogen). Daudi cells (DPA1*010301; 020101 and DPB1*020102; 0802) were maintained in RPMI with the above supplements. MeJuso cells were typed by the NHS Blood and Transplant Unit (Newcastle, UK) to be homozygous for DPB1*1301.

The mAbs 1B5 against DR\(\alpha\) and HC10 against MHC class I, and the polyclonal anti-sera against DR\(\beta\), DQ and DP were a gift from Prof. J. Neefjes (NKI, Amsterdam, The Netherlands). The polyclonal DP serum predominantly recognises the DP\(\beta\) chain and only weakly recognises the DP\(\alpha\) chain when DP\(\alpha\beta\) are co-expressed. The mAb against the li (PIN.1) and mAbs HL40 (anti-DR\(\beta\)/DP\(\beta\)), HL37 (anti-DQ\(\beta\)) and KUL/05 (anti-class II\(\beta\)) were purchased from Abcam. The anti-DQ mAbs L2 and SPV-L3 were a kind gift from Prof. J. Robinson (Newcastle, UK).

*Constructs*

The DM constructs have been previously described (20). The li (short isoform) and DR1 constructs (DRA*0101 and DRB1*010101) were a kind gift of Prof. J. Neefjes (NKI, Amsterdam, The Netherlands). The DQ and DP constructs in pCMV6 were obtained from Origene: DQA1*010202, DQB1*050101 (DQ5), DPA1*010301, DPB1*040101 (DP4) and DPB1*1701. The HLA-DPB cysteine to alanine mutants (C211A, C15A/C77A) were generated using Quik Change Site-Directed Mutagenesis kit (Stratagene). Each sequence was confirmed by DNA sequencing.

*Transfections*

Transfections were done with Lipofectamine 2000 (Invitrogen) or Fugene (Roche) according to the manufacturer’s instructions. For Lipofectamine transfection sub-confluent cells in 6-cm dishes were washed with HBSS and OptiMem and transfected with 1 \(\mu\)g of DNA for 6 h in the presence of OptiMem serum-free medium. After 6 h the cells were washed and placed back in normal growth medium. For Fugene transfections, the transfection mix was added to the medium. The cells were analyzed 24 h post-transfection and expression of all chains was confirmed by Western blotting. Where indicated, cells were incubated with
leupeptin (15 μM), NH₄Cl (20 mM) or vehicle control 1 hour before transfection until lysis.

**Immunostaining**
Cells grown on coverslips were fixed in 4% paraformaldehyde in PBS for 10 minutes and were either left untreated, or were incubated in 0.2% Triton X100 in PBS to permeabilise the cells. After blocking in 0.2% BSA in PBS, the cells were incubated with primary antibodies for 1 hr, washed in 0.2% BSA/PBS, and incubated with fluorescently labelled secondary antibodies for 1 hr (Alexa 488, Invitrogen). The nuclei were stained with DAPI before mounting the coverslips with Vectashield (Vector Laboratories). Images were taken on an Axio imager.M1 with OpenLab software.

**Immunoprecipitations and Western blotting**
Cells and transfectants were lysed on ice with lysis buffer (1% Triton X100, 50 mM TrisHCl pH8.0, 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail (1 μg/ml of antipain, chymostatin, leupeptin, pepstatin A)). Post-nuclear lysates were incubated with protein A Sepharose beads (Sigma/Amersham) and antibodies for 1-2 h at 4ºC. After extensive washing of the beads, immunoprecipitated proteins were eluted by boiling in sample buffer and analyzed by 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) at 150 mA for 2 h. The membranes were blocked in Tris-buffered saline Tween (TBST) with 8% milk, followed by incubation with primary antibody. After washing 3 times with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (DAKO), washed and visualised by ECL (Amersham) and exposure to film (Kodak). Protein markers were from Bio-Rad.

**SDS-stability assay**
Lysates were 1:1 diluted with 2x reducing sample buffer (4% SDS, 20% glycerol, 120 mM TrisHCl pH 6.8, bromophenol blue, 100 mM DTT). Half of the sample was left at room temperature, while half of the sample was boiled for 5 minutes, before analysis by SDS-PAGE and Western blotting as described above. These experiments were repeated 2-5 times with reproducible results.

**RESULTS**

**Reconstitution of HLA-DR, -DQ and -DP.**
We and other investigators have shown that different non-APCs can be reconstituted with a functional MHC class II compartment by transfection of HLA-DR molecules, the li and DM (20-22). The advantages of this system are that folding, assembly and trafficking of different MHC class II isotypes can be compared in the same cell line, and that the contribution of individual proteins (li, DM and class II αβ chains) can be examined in the absence of competing endogenous proteins. To assess whether the different MHC class II isotypes, HLA-DR, -DQ and -DP would all assemble in non-APCs, we transfected HeLa cells with different combinations of α, β, and li constructs. Lysates were subjected to immunoprecipitation with 1B5 (DR), HL37 (DQ) or HL40 (DP). 1B5 efficiently immunoprecipitated the DR α chain (Figure 1A, lanes 3/4) and co-immunoprecipitated DR β and the li (Figure 1A, lane 7/8 and lane 12, respectively). 1B5 did not aspecifically co-immunoprecipitate the DR β chain or li (Figure 1A, lanes 6 and 9 respectively). HL37 directly immunoprecipitated the DQ β chain (Figure 1B, lane 2) and co-immunoprecipitated DQ α and the li (Figure 1B, lane 3 and 6, respectively). HL37 did not immunoprecipitate DQ α directly (Figure 1B, lane 1) and association with the li was dependent on expression of both DQ α and DQ β (Figure 1B, compare lanes 5 and 6). The mAb HL40 directly immunoprecipitated DP β (Figure 1C, lanes 2) but did not aspecifically immunoprecipitate the li (Figure 1C, lane 4). HL40 co-immunoprecipitated DP α and the li from DPαβ/li transfectants (Figure 1C, lane 3/5). We conclude that HLA-DR, DQ and DP could all be effectively reconstituted in HeLa cells.

**Ii is required for endosomal-lysosomal deposition of HLA-DR, -DQ and -DR.**
Previous studies have shown that endosomal-lysosomal deposition of HLA-DR is critically dependent on the li (23). To determine whether HLA-DQ and -DP are equally dependent on the li for intracellular localisation we used immunofluorescence microscopy (Figure 2). HeLa cells were transfected with β chain only, or in combination with the α chain, li or DM. The cells were fixed and either left unpermeabilised (-Tx, to demonstrate cell surface deposition), or were permeabilised (+Tx,
to show intracellular distribution) before immuno-staining with HL40 (DR and DP) or HL37 (DQ). Single β chains showed a typical ER staining (Figure 2A-C, +Tx) and were not detected at the plasma membrane (-Tx). When the α chain was co-transfected with the β chain, HLA-DR, -DQ and -DP were still mainly localised in the ER (Figure 2A-C, +Tx), however cell surface expression was observed on non-permeabilised cells (Figure 2A-C, -Tx). This indicates that a proportion of αβ dimers were able to reach the cell surface. Co-expression of the Ii with αβ dimers, however, resulted in a punctate staining indicative of endosomal-lysosomal localisation (Figure 2A-C, +Tx), similar to that seen in professional APCs. Quantification of repeat experiments showed that of ~70 cells counted, 80% (DP), 93% (DR) and 86% (DQ) of αβ+Ii+DM expressing cells were positive for the appearance of endosomal-lysosomal structures, as expected from previous experiments with DM (20). DP, DQ and DR were all able to rescue the known “swollen endosomal-lysosomal vesicle” phenotype that arose when cells expressed Ii alone (supplementary Figure 1; (24)). In the presence of the Ii, MHC class II molecules were readily observed at the cell surface (Figure 2A-C, -Tx). Additional expression of DM did not change the intracellular and cell surface expression of MHC class II molecules (Figure 2A-C, right panels). Thus, MHC class II αβ dimers of all subtypes require the Ii to reach the endosomal-lysosomal system. Cell surface expression of HLA-DR, -DQ and -DP in the absence of the Ii is consistent with previous observations (23,25-27). The result is not likely to be an artefact of overexpression, since expression levels were similar to or lower than those found in APCs (20) and the β chain alone did not reach the cell surface.

Thus, not only DR, but also DQ and DP interacted physically (Figure 1) and functionally (Figure 2) with Ii.

**Differences in the formation of SDS-stable HLA -DP, -DQ and -DR dimers.**

The acquisition of a stable binding peptide results in a conformational change that renders HLA-DRαβ dimers resistant to dissociation in SDS-containing sample buffer at room temperature (28). To compare the assembly of HLA-DR (DR1) with HLA-DQ (DQ5) and –DP (DP4), we made use of this well-established SDS-stability assay. We co-transfected MHC class II α and β chains with different combinations of Ii and DM. The expression levels of Ii and DM were confirmed by western blotting (not shown). To analyse DP, DQ and DR, lysates in SDS-containing sample buffer were left at room temperature or were boiled. As expected, formation of SDS-stable DRαβ dimers required expression of both the Ii and DM (using the anti-DRα Mab 1B5; Figure 3A, lanes 5 and 6). Expression of either the Ii or DM alone was not sufficient to induce the formation of SDS-stable DR dimers (Figure 3A, lanes 3/4 and 7/8; see also van Lith and Benham Figure 5 (20).

In contrast, the requirements for HLA-DQ to become SDS-stable were different than those for HLA-DR. Co-expression of the Ii alone resulted in SDS-stable DQαβ dimers (using a polyclonal anti-DQ serum; Figure 3B, lanes 3/4). Additional expression of DM increased the amount of SDS-stable DQαβ dimers (Figure 3B, lanes 5/6), indicating that DM promoted peptide loading of HLA-DQ. Co-expression of DM in the absence of the Ii resulted in a negligible amount of dimers (Figure 3B, lanes 7/8), probably because DQαβ did not intersect with lysosomal loading compartments without the Ii (Figure 2B). To confirm that DQ could form SDS-stable dimers with co-expression of the Ii only, we used two additional anti-DQ mAbs, L2 and SPVL3. Both L2 and SPVL3 clearly detected DQ dimers when co-expressed with the Ii (Figure 3C, lanes 1/2 and 3/4) but did not recognise DQαβ dimers when only α and β chains were expressed (Figure 3C, lanes 1/2 and 5/6). The blot was co-probed with HC10 (detecting MHC class I; cI.I) to confirm equal loading of the samples.

In stark contrast to HLA-DR1 and – DQ5, HLA-DR4 was not dependent on either the Ii or DM to form SDS-stable dimers (using a polyclonal DP antiserum; Figure 3D, lanes 1/2). Ii alone facilitated the formation of slightly more compact αβ dimers (Figure 3D compare lanes 2 and 4). DM alone also facilitated SDS-stable DP dimer formation (Figure 3D, lanes 7/8), indicating that the targeting of DP to the lysosomal system was not required for its stability. Co-expression of Ii and DM further increased the amount of SDS-stable DP dimers (Figure 3D, lanes 5/6). Thus either Ii or DM alone may facilitate the transition of HLA-DP to a more compact dimer state. Note that the
amount of monomeric DQ and DP detected before and after boiling remained similar, whereas the amount of DRα detected after boiling increased. This may be because the DP and DQ antibodies detect monomers less efficiently than 1B5.

The DP serum also detected a background band at 50kD (*, Figure 3D), the intensity of which varied between experiments. However, the presence of the background band did not influence the recovery of stable DPαβ dimers. As shown in Figure 3E, when the background band was absent, stable DP dimers formed between the DPαβ chains alone, and these became more compact in the presence of Li. This experiment also shows that DPβ chains alone did not form SDS-stable dimers. In addition, incubation of semi-permeabilised HLA-DP transfectants with a specific DP binding peptide resulted in an increased recovery of stable dimer, showing that DP molecules could be stabilised by peptide in this system (supplementary Figure 2 and supplementary information).

To see whether the unusual stability of DP4 was shared by other DP molecules, we tested DPB1*1701, a beryllium disease-associated allele. This allele also gave SDS-stable dimers in the absence of the Ii and DM (Figure 4A, lane 2). The amount of stable DPB1*1701 αβ dimers increased with co-expression of the Ii, further demonstrating that DP stability was unusual in that it did not require DM (Figure 4A, lane 6).

The observed stability of DPαβ might reflect epitope(s) specifically detected by the anti-DP serum. To examine whether a different antibody could detect SDS-stable DP dimers, we used the DP-reactive Mab KUL/05 (29). KUL/05 recognised DPβ monomers and DP dimers in DPαβ transfectants, demonstrating that the detection of DP stability was not an antibody specific phenomenon (Figure 4B, lanes 3/4). The amount of dimers increased when DPαβ was co-transfected with the Ii and DM, as expected (Figure 4B, lanes 5/6). The DP dimers in transfectants were similar to those in MelJuso, a cell line that expresses MHC class II molecules endogenously (Figure 4C, lanes 5-8). Note that the recognition of DP monomers decreased in cells expressing endogenous DP (Figure 4C, lanes 7-8), but that both dimers and monomers could be detected by the DP antiserum after immunoprecipitation from MelJuso with the HL40 mAb (Figure 4C, lanes 11/12).

Taken together, these data demonstrate that a pool of HLA-DPαβ molecules, unlike HLA-DR1 and –DQ5, can assemble in the absence of the Ii and become SDS-stable without intersecting the lysosomal pathway.

Oxidative assembly of HLA-DR, DQ and DP in the ER.

The differences in the SDS-stability of DR, DQ and DP could result from differences in the folding and intrinsic stability of the class II molecules. Having observed that oxidative protein folding is important for hetero-dimeric (and Ii independent) assembly of DM (20), we compared the disulfide-dependent protein oxidation of HLA-DR, -DQ and –DP (Figure 5). Lysates from HLA-DR, -DQ and -DP transfectants were analyzed by Western blotting after non-reducing SDS-PAGE to preserve intra- and intermolecular disulfide bonds. The “non-reducing” complexes observed in these experiments were obtained after boiling in sample buffer –DTT, and are therefore not the same as those observed in Figures 3 and 4, which are obtained without boiling in sample buffer +DTT.

All β chains migrated faster under non-reducing conditions (Figure 5B-D), indicating the presence of intra-molecular disulfide bonds. This was regardless of whether β was co-expressed with α chains or accessory proteins. In contrast, the α chains migrated similarly under reducing and non-reducing conditions (Figure 5A/C/D, compare R versus NR), although note that the DQ and DP antisera only weakly recognised the α chains, especially under non-reducing conditions. The difference between α and β chains in migration under non-reducing conditions is explained by the presence of two long-range disulfide bonds in β chains versus one in α chains, which makes the β proteins more compact under non-reducing/denaturing conditions.

Despite these general similarities for DR, DQ and DP, there are some marked differences. Unlike the DQβ monomers, the DRβ and DPβ monomers existed in two distinct oxidation states (Figure 5B-D, top panels, bands 1 and 2 for DR and DP). This may be explained by the presence of additional cysteine residues in DRβ and DPβ. The DRα chain also appeared as a doublet, but this did not reflect different
oxidation states, as the pattern was essentially the same under reducing conditions (Figure 5A, lanes 3-5). Rather, the DRα doublet is most likely due to two differently glycosylated pools (30), similar to that seen for DMα (20).

The non-reducing gels also revealed differences in disulfide-linked complexes across the 3 MHC class II molecules. In contrast to DR and DP, DQ hardly formed any disulfide-linked complexes, which may reflect the absence of any unpaired cysteine residues in this molecule (Figure 5C). The disulfide-linked DRα and DRβ dimers and higher m.w. complexes gradually diminished with further reconstitution (Figure 5A/B, top panels). All dimers and high molecular weight complexes disappeared under reducing conditions (not shown; see also Figure 3A-D lanes 1, 3, 5, 7). DPα and β formed complexes at ~50 kD (Figure 5D): a lower one when DPβ was expressed alone (lane 2, band 3), and an additional higher one when DPα was co-expressed (lanes 3-5, band 4). Therefore, band 3 probably represented DPβ disulfide linked dimers, while band 4 could represent DPαβ disulfide-linked dimers, as they were only present when DPα was co-expressed. The presence of band 4 in DPαβ transfectants (and in combination with Ii and DM) correlated with the requirements for DP SDS-stability (Figure 3D) and raised the possibility that a disulfide-bonded complex between DPαβ might be responsible for DP stability in the absence of the Ii.

To exclude that disulfide-linked DPαβ dimers accounted for the SDS-stability of DP in DPαβ transfectants, we made use of a single cysteine mutant of DPβ, C211A. In contrast to wild-type DPβ, this mutant did not generate band 4 when co-expressed with DPα (Figure 6A, lanes 3/4). This mutant, however, was still able to form SDS-stable dimers when DPα and DPβ C211A were co-expressed (Figure 6B, lanes 9/10). To further confirm the molecular nature of the DPαβ dimer, we constructed a DPβ mutant that lacked the conserved C15/C77 disulfide bond. The C15/C77 mutant did not form SDS-stable complexes with the DPα chain (Figure 6B, lanes 5/6 and 11/12) demonstrating that disulfide stabilization of the peptide binding site was required for αβ complex formation, and additionally confirming the specificity of the DP antiserum. The higher expression levels of the C211A mutant suggest that this C-terminal cysteine may be involved in degradation of orphan DPβ chains (31), or required for interaction with cytosolic/membrane components for DP transport (32); this will be explored in subsequent work. Taken together, these experiments show that the DP SDS-stable dimers observed in Figure 3 were not the result of misoxidised proteins.

**HLA-DP SDS-stable dimers are leupeptin insensitive.**

Having shown that the Ii is not absolutely required for the stability of properly folded DP, we wanted to establish whether DP stability is acquired in the endosomal-lysosomal system. Formation of SDS-stable HLA-DR dimers can be prevented by treatment of cells with the cysteine.serine protease inhibitor leupeptin (33). Leupeptin prevents the complete degradation of the Ii and affects the generation of peptides by leupeptin-sensitive proteases (34). To see whether the Ii/DM independent formation of SDS-stable DPαβ dimers is sensitive to leupeptin, cells were treated with leupeptin and transfected with different combinations of DRαβ, DPαβ, Ii and DM. After 24 hours, lysates from the transfectants were subjected to the SDS-stability assay. As expected, DRαβ in the absence of the Ii did not gain SDS-stability (Figure 7A, lanes 1-4). When cells co-expressed the Ii and DM, DR clearly formed SDS-stable dimers (Figure 7A, lanes 5/6), which were almost completely abrogated by leupeptin treatment (Figure 7A, lanes 7/8). DP dimers were easily detected in DPαβ transfectants and these were not disrupted by leupeptin treatment (Figure 7B, lanes 5-8). As expected, DPβ alone did not form dimers (Figure 7B, 1-4). Our results show that unlike DR, DP molecules can acquire SDS-stability by an Ii/DM independent pathway that is insensitive to leupeptin.

To examine whether leupeptin-insensitive DP complexes existed in antigen presenting cells, we investigated the behaviour of DR and DP in a melanoma cell type (MelJuso) and a lymphoma cell line (Daudi) that endogenously express MHC class II molecules. Remarkably, leupeptin or NH4Cl (which neutralizes lysosomal pH) treatment left DP dimers intact (Figure 8B/D), whereas DR1 dimers from the same lysates were lost (Figure 8A/C). Thus, unlike DR1, a pool of DP molecules can acquire stability outside the
classical endosomal/lysosomal pathway in both transfectants and in professional antigen presenting cell types.

**DISCUSSION**

In this paper, we have directly compared the assembly and stability requirements for HLA-DP, -DQ and -DR for the first time. We show that HLA-DR, -DQ and -DP differ markedly in their requirements for the invariant chain and DM, despite having ~70% amino acid sequence similarity. Our results show that HLA-DR, -DP and -DQ all require the Ii for endosomal-lysosomal targeting (Figure 2) but not for stability (Figure 3). Although αβ complexes are ER localised in the absence of DM or Ii, at least a portion of these αβ complexes are folded and can exit the ER, bypassing the endosomal-lysosomal system. The concept that the Ii is not required for the quality control of DP, DQ or DR per se is supported by work in the mouse, where residual MHC class II molecules appear at the cell surface in the absence of the Ii (25) or when class II is transfected in the absence of the Ii (26,27). The effect of Ii deficiency in mice is also allele specific; for example, the Balb/c Ii knockout has a mild phenotype and develops functional CD4+ T cells (35). In the absence of functional Ii, H-2b does not assemble properly in spleen cells, but H-2k is unaffected (36). In H-2k mice, loss of DM has an effect on E(k) but not A(k) class II molecules (37), supporting the notion that the need for the MHC class II chaperones is allele-dependent. In vivo, Ii gene expression is not absolutely co-ordinated with MHC class II synthesis (38), and there are circumstances where deregulation of Ii may occur, for example during HIV infection, where Ii is a target of Nef (39). Our results therefore raise the possibility that expression levels of Ii could be exploited to manipulate the relative levels of stable MHC class II molecules, either by pathogens, or for therapeutic benefit.

Although there are no published studies on the effect of Ii and DM on DP antigen presentation, our finding that the Ii is sufficient for DQαβ to attain an SDS stable conformation is supported by Ettinger et al. who suggest that DQ0602 may present antigen in a DM independent fashion (15). We demonstrate here that the Ii alone is required for recognition of SDS-stable DQ5 dimers by L2 and SPVL3 (Figure 3C). The observation that DQ5 (and DP) can be stabilised in the absence of DM (Figure 3) has implications for the function of HLA-DO. DO regulates DM activity in a pH dependent manner (40). It will be interesting to test whether DO can selectively adjust DP or DQ peptide binding, resulting in different relative expression of HLA-DR, DQ and DP on different APCs.

For MHC class I molecules, it has been suggested that the C-terminal cysteine residue of HLA-B7 can influence recognition by NK cells (41). We noted that DPβ chains have an unpaired C211 residue that is not shared with DR and DQ β chains, leading us to investigate whether C211 could also influence conformation at a spatially distant site. The DPβ C211 residue is not required for DP SDS stability although C211 does increase the propensity of DPβ to form disulfide linked complexes (Figures 5 and 6). In contrast, the C15-C17 disulfide bond, which anchors the peptide-binding domain, is required for the stable assembly of DPβ with its cognate α chain (Figure 6).

SDS stability is a well-documented readout for functional, peptide loaded HLA-DR complexes (28). Here, we have shown unexpectedly that HLA-DP4 does not need Ii to acquire this property (Figures 3, 6 and 7), and that this is not allele specific, antibody specific (Figure 4) or cell type specific (Figure 8). Given that DR1 molecules can be stabilised in vitro with short peptides (42), it will be important to establish whether the stable DPeβ complexes seen in different conditions are empty, loaded with peptides or a mix of the two. Although our experiments show that DP in semi-permeabilised transfectants can be stabilised by a specific antigenic peptide (supplementary Figure 2), it remains possible that an unknown accessory factor, such as an ER chaperone, might help to stabilise “empty” DP complexes. In vitro assays have shown that DP can certainly bind to CLIP fragments (43) and known HLA-DP peptide-binding motifs differ from those of (ER-loaded) MHC class I molecules, so DP is not likely to compete for classical class I-binding peptides (44). However, peptide elution studies have demonstrated that HLA-DP2 is naturally loaded with ER protein derived peptides, including ERP57 (PDIA3) and Grp94 (endoplasmin) (45), suggesting that in vivo some DP molecules loaded with ER peptides reach the cell surface. It will also be important to establish
whether peptides in the ER can compete with Ii to load DPαβ in APCs, or whether peptides from viral and ER proteins are actually obtained at the cell surface or during DP recycling. The possibility that proteases at the plasma membrane, or in the extracellular matrix, play a role in DP peptide loading in vivo deserves further exploration. Another possibility is that the relative Ii independence of DP makes it more accessible to peptides during autophagy (46), which might also explain why DP cross-presents viral antigens.

One of the first reports about DP (then named SB) function was on the presentation of viral, rather than bacterial, antigens, namely from herpes simplex and influenza viruses (47). It was further demonstrated that SB/HLA-DP, when transfected into murine fibroblasts, could present influenza viral peptides to DP restricted human T cells (48). There are a growing number of examples of viral peptides that bind to DP molecules and elicit CD4+ T cell responses, particularly for DP4, the allele used in this study. Of particular note is the differential association between DP and chronic Hepatitis B in Asian populations (49). It will be informative to compare peptide binding and assembly of different susceptibility haplotypes with the behavior of the protective DP4 examined in our study. Further exploration of the molecular details of DP conformation and stability may shed light on why some autoimmune diseases are DP linked, and whether unique therapeutic routes for peptide delivery to DP can be exploited.

REFERENCES

FOOTNOTES

We thank John Robinson, Andrew Knight and Jacques Neefjes for helpful discussions and reagents. This work was supported by the BBSRC grant BBC509582.

FIGURE LEGENDS

Figure 1. Reconstitution of HLA-DR, -DQ and -DP in a non-APC cell line. HeLa cells were transfected with different combinations of α, β and Ii constructs as indicated. Lysates were first subjected to immunoprecipitation with 1B5 (A), HL37 (B) or HL40 (C), before Western blotting with 1B5, anti-DRβ and anti-Ii (A), anti-DQ and anti-Ii (B) and anti-DP and anti-Ii (C). H and L indicate the heavy and light chains of the antibodies used for the immunoprecipitation. Note that the HL37 antibody light chain migrates more slowly than the HL40 antibody light chain.

Figure 2. Chaperone requirements for intracellular trafficking of HLA-DR, -DQ and -DP. HeLa cells were transfected with DR (A), DQ (B) or DP (C) with β chain alone or in the combinations indicated (top). The cells were fixed in paraformaldehyde and either left unpermeabilised (-Tx), or were permeabilised with 0.2% Triton X100 (+Tx). DR and DP transfected cells were immuno-stained with mAb HL40, while DQ transfected cells were immuno-stained with mAb HL37. Nuclei were stained with DAPI.

Figure 3. DP gains an SDS-stable conformation in the absence of Ii or DM. The α and β chain of DR, DQ and DP were transfected into HeLa cells, either alone or in combination with the Ii and DM as indicated. Lysates were subjected to the SDS-stability assay, followed by SDS-PAGE and Western blotting with 1B5 (A, anti-DRα), anti-DQ (B), L2+HC10 (C, left panel), SPVL3+HC10 (C, right panel) or anti-DP (D,E). The positions of the monomeric α and β chains are indicated as well as the SDS-stable dimers. Note that the polyclonal DP anti-serum only weakly recognises DPα. Asterisks indicate background bands that are also present in mock transfectants (data not shown). c.I = MHC class I.

Figure 4. SDS-stable DP dimers are not allele or antibody specific. HeLa cells were transfected with different combinations of DPαβ chains and Ii as indicated. Lysates were subjected to the SDS-stability assay, followed by SDS-PAGE and Western blotting with anti-DP (A and C) and KUL/05 (B). A MelJuso lysate was subjected to immunoprecipitation with HL40 before detection with anti-DP (C, lanes 11-12). The positions of the monomeric α and β chains are indicated as well as the SDS-stable dimers. Note that DPβ1701 migrates more slowly than DPβ0401 because of charge differences and KUL/05 does not recognise monomeric α chains. Asterisks indicate background bands.

Figure 5. Disulfide bonds in HLA-DR, -DQ and -DP. HeLa cells were transfected with different combinations of α, β, Ii and DM. Lysates were analyzed by reducing (R, bottom panels) and non-reducing (NR, top panels) SDS-PAGE, and Western blotting with 1B5 (anti-DRα, A), anti-DRβ (B), anti-DQ (C) and anti-DP (D). The positions of monomeric α and β chains are indicated on the left. Note that the polyclonal anti-sera against DQ and DP weakly detect the α chains (especially under non-reducing conditions). Bands 1 and 2 represent two oxidation states in monomeric DRβ and DPβ. Bands 3 and 4 represent likely disulfide-linked DPβ homodimers and disulfide linked DPαβ heterodimers, respectively.
Figure 6: The C15-C77 β chain disulfide bond is required for DPαβ SDS-stability. Wt DPβ, DPβ C211A or DPβ C15A/C77A were transfected into HeLa cells, either alone or in combination with DPα. Lysates were analyzed by non-reducing (NR) SDS-PAGE (A) or by reducing SDS-PAGE with and without boiling in sample buffer (B) followed by Western blotting with anti-DP. Bands 3 and 4 represent likely disulfide-linked DPβ homodimers and disulfide linked DPαβ heterodimers respectively. Asterisks indicate background bands.

Figure 7: Formation of DPαβ heterodimers is leupeptin insensitive. Different combinations of α, β, Ii and DM were transfected into HeLa cells mock-treated or treated with leupeptin. Lysates were subjected to the SDS-stability assay, followed by SDS-PAGE and Western blotting with 1B5 (A, anti-DRα) or anti-DP (B). The positions of the monomeric α and β chains are indicated as well as the SDS-stable dimers. Asterisks indicates background band.

Figure 8. Endogenously expressed DR and DP are differentially sensitive to treatment with leupeptin and NH₄Cl. MelJuso and Daudi cells were incubated in the presence of 15 μM leupeptin or 20 mM NH₄Cl for 48 hours. After lysis, the samples were analyzed with the SDS-stability assay. MelJuso 1B5 (A); MelJuso anti-DP (B); Daudi 1B5 (C); Daudi anti-DP (D). Note the weak recognition of DP monomers in cell lines expressing endogenous DP, and the slower migration of DRα chains compared to DP monomers. D=SDS-stable dimers, M=monomers.
Figure 1

A  IP: 1B5 (αDRα)

B  IP: HL37

C  IP: HL40
Figure 2
Figure 3

A

\[
\begin{array}{cccccccc}
\alpha\beta & \alpha\beta & \alpha\beta & \alpha\beta \\
B & NB & B & NB \\
dimer & DR\alpha & DR\alpha & DR\alpha \\
1 & 2 & 3 & 4 \\
WB: 1B5 & WB: 1B5 & WB: 1B5 & WB: 1B5 \\
& 5 & 6 & 7 & 8
\end{array}
\]

B

\[
\begin{array}{cccccccc}
\alpha\beta & \alpha\beta & \alpha\beta & \alpha\beta \\
B & NB & B & NB \\
dimer & DQ\alpha & DQ\alpha & DQ\alpha \\
1 & 2 & 3 & 4 \\
WB: \alpha DQ & WB: \alpha DQ & WB: \alpha DQ & WB: \alpha DQ \\
& 5 & 6 & 7 & 8
\end{array}
\]

C

\[
\begin{array}{cccccccc}
DQ & DQ & DQ & DQ \\
B & NB & B & NB \\
dimer & cl.l & cl.l & cl.l \\
1 & 2 & 3 & 4 \\
WB: L2+HC10 & WB: SPVL3+HC10 & WB: SPVL3+HC10 & WB: SPVL3+HC10 \\
& 5 & 6 & 7 & 8
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D

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dimer & D\alpha & D\alpha & D\alpha \\
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& 5 & 6 & 7 & 8
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E

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\begin{array}{cccccccc}
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B & NB & B \\
dimer & \beta & \beta & \beta \\
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WB: \alpha DP & WB: \alpha DP & WB: \alpha DP & WB: \alpha DP \\
& 5 & 6
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Figure 4

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Figure 5
Figure 6

A

B

WB: αDP (NR)

DPα+

DPβ

DPβ C211A

DPβ C211A

1 2 3 4

250

150

100

75

50

37

25

20

DPβ

DPβ C15A

DPβ C77A

DPβ C211A

DPβ C77A

1 2 3 4 5 6 7 8 9 10 11 12

dimer

* DPβ

WB: αDP

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Figure 7

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WB: 1B5 (αDRα)

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WB: αDP
Figure 8
HLA-DP, HLA-DQ and HLA-DR have different requirements for invariant chain and HLA-DM
Marcel van Lith, Rosanna M. McEwen-Smith and Adam M. Benham

*J. Biol. Chem.* *published online* October 19, 2010

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