A Mutant Human Histocompatibility Leukocyte Antigen DR Molecule Associated with Invariant Chain Peptides

By Elizabeth Mellins,* Patricia Cameron,† Miguel Amaya,* Steven Goodman,* Donald Pious,$ Laura Smith,$ and Benjamin Arp$ 

From the *Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104; †Merck Research Laboratories, Rahway, New Jersey 07065; and the $Department of Pediatrics, University of Washington, Seattle, Washington 98195

Summary

From a human histocompatibility leukocyte antigen (HLA)-DR/DQ hemizygous, B lymphoblastoid progenitor, we isolated a cell line, 10.24.6, with a DRα missense mutation (96P→96S), which results in an N-linked carbohydrate addition at position 94 in the DRα2 domain. Several features of 10.24.6 cells suggest that the mutation disrupts normal intracellular formation of peptide/DR complexes. The mutant HLA-DR dimers, though expressed at the cell surface, lack the conformation of the mature, peptide-loaded class II molecules of the progenitor cell, as assessed by their loss of binding of certain antibodies and by the lack of stability in detergent (sodium dodecyl sulfate) solution. In addition, presentation of endocytosed antigen to HLA-DR-restricted T cells is defective in the mutant, but can be restored by transfection of a wild type DRA gene. Assays with synthetic peptides indicate that the 10.24.6 phenotype is not due to an intrinsic inability of the mutant DR molecules to bind peptides. Therefore, to directly evaluate peptide occupancy of the mutant molecules, we analyzed acid-eluted, HLA-DR-associated peptides. The predominant species from the 10.24.6 mutant is a nested set of invariant chain (II)-derived peptides that are undetectable in the DR eluate from progenitor cells. The region of DRα altered in the mutant molecules is thus implicated in normal formation of peptide/DR complexes. Further, the same set of II peptides associated with the DR molecules is present in the eluate from an antigen presentation mutant with a defect in an major histocompatibility complex (MHC)-linked gene. These results suggest that DR molecules in 10.24.6 and in certain presentation mutants are affected at the same or related steps in class II molecule biosynthesis, raising the possibility that class II molecules interact with an MHC-encoded accessory molecule during antigen presentation.

Class II MHC molecules are heterodimeric (α/β) transmembrane glycoproteins. They are expressed at the surface of antigen-presenting cells (macrophages, dendritic cells, B cells), where they present bound peptides for surveillance by CD4+ T cells. Peptides that become bound to class II molecules are generated by processing events within the endocytic pathway, and the predominant class II-associated peptides derive from membrane and extracellular proteins (1, 2). Detection of peptide/class II molecule complexes with specific T cells suggests that some peptides derived from other sources also become associated with class II molecules (3, 4).

The formation of intracellular complexes between MHC class II molecules and peptides is a regulated process. One molecule involved is the invariant chain (II), which associates with nascent class II molecules and mediates their localization to endocytic compartments through a sequence in its cytoplasmic tail (5, 6). II cleavage and dissociation are apparent prerequisites for class II molecule/peptide binding (7). Expression of the p41 form of II, which has an alternatively spliced intraluminal portion, increases efficiency of presentation of certain antigens, perhaps by further modulation of class II molecule trafficking (8). A second regulatory molecule is implied by data from mutant B lymphoblastoid (B-LCL)1 cell lines that are defective in class II-restricted antigen presentation, but express normal levels of both invariant chain and class II molecules (9). The defect in these

1 Abbreviations used in this paper: B-LCL, B-lymphoblastoid; C2P-1, class II presentation locus; HBsAg, hepatitis B surface antigen; MOMP, major outer membrane protein; PPD, purified protein derivative; TI, tetanus toxoid.
mutants affects generation of peptide/class II molecule complexes from endocytosed proteins, resulting in deficient presentation of intact protein antigens (though not of synthetic peptides) and in altered conformation of class II molecules expressed at the cell surface. The gene responsible for this phenotype (hereafter called "class II presentation locus" [C2P-1]) maps to the MHC (10-12), but its identity and specific function are unknown.

Analyses of the relationship of fine structure to function in MHC class II molecules have delineated regions or residues involved in T cell receptor recognition (summarized in reference 13), peptide binding (13, 14), interaction with the CD4 molecule (15, 16), and most recently, class II dimer–dimer interactions (14). However, regions of MHC class II molecules specifically related to peptide loading have not been mapped. To elucidate structure–function relationships in human class II molecules, we have isolated informative mutant B-LCLs (17, 18). Here, we describe a novel B-LCL, 10.24.6, in which HLA-DR molecules with an α2 domain mutation fail to become appropriately loaded with naturally processed peptides, even though the mutant molecules are capable of binding DR-restricted peptides in vitro and the class II molecule loading pathway is intact in the cells. Our findings indicate that normal intracellular formation of peptide/HLA-DR complexes involves the region of the DR molecule altered by the 10.24.6 mutation. Further, as the phenotype of the mutant DR molecules resembles that of DR molecules in a C2P-1 mutant derived from the same progenitor, our findings suggest that class II molecules and the C2P-1 regulatory protein may directly interact.

Materials and Methods

**Antigen-presenting Cell Lines.** The progenitor cell line, 8.1.6, is a DR/DQ hemizygous B-LCL that expresses the HLA class II specificities DR3, DRw52a, DQ2, and DP4.1, DP4.1 (reference 17). To derive mutant 10.24.6, a young clone of 8.1.6 was mutagenized with ethyl methane sulfonate and immunoselected with mAb 16.23 plus complement, as described (see reference 17 for details). Mutants 9.5.3, 9.10.3, and 10.53.6 are C2P-1-defective mutants (see text), which were also derived from progenitor 8.1.6 by immunoselection with mAb 16.23; the phenotypic characteristics of these mutants have been previously described in detail (9). 9.22.3 is a DR-null B-LCL, derived from 8.1.6 by deletion of the single DRA gene in 8.1.6 (reference 17); 22.9.23 retains expression of HLA-DQ and -DP molecules and presents DQ- and DP-restricted antigens normally (19). To generate the 10.24.6 × 9.22.3 hybrid, dominant selectable markers were introduced into 9.22.3 and 10.24.6. An Escherichia coli gpt gene, conferring resistance to mycophenolic acid, was transactivated into 9.22.3 by electroporation as described (20). The dihydrofolate reductase gene, conferring resistance to methotrexate, was introduced into 10.24.6 by cotransfection with PA317-SDHt, a retrovirus packaging fibroblast line, provided by A. Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, WA. Somatic cell hybrids were prepared by polyethylene glycol (PEG) fusion, as described (9). For generation of the 10.24.6-DRA transfectant, 10.24.6 was cotransfected with PA317LHC-DRAc6, a retrovirus packaging fibroblast line containing the DRA cDNA and the hygromycin phosphoribosyl transferase gene (21); this cell line was generically provided by William Kwok, Virginia Mason Research Center, Seattle, WA. Transfected 10.24.6 cells were selected in 100 µg/ml hygromycin B (Sigma Chemical Co., St. Louis, MO) and cloned in soft agar.

**T Cells and T Cell Proliferation Assays.** The DR3-restricted T cell clone specific for residues 241-265 of the major outer membrane protein (MOMP) of Chlamydia trachomatis was isolated as described (Barbosa, J., manuscript submitted for publication) and kindly provided by Dr. James Barbosa, Miles, Inc., West Haven, CT. The other antigen-specific T cell clones and lines were isolated and characterized as described (9, 19). The alloreactive, anti-DR3 clone was isolated as described (22) and was the gift of Dr. Armead Johnson, Georgetown University, Washington, DC. The alloreactive, anti-DQ2 clone (Cotner, T., unpublished results) was the gift of Dr. Tom Cotner, University of Washington, Seattle, WA. The alloreactive, anti-DP4 T cells were isolated as described (23) and were kindly provided by Susan Masewicz, Fred Hutchinson Cancer Research Center. None of the alloreactive cell lines respond normally to 8.1.6-derived, presentation defective (C2P-1) mutants as stimulators (24 and Mellins, E., unpublished results). For assay, 2 × 10⁵ T cell blasts were cocultured with 10⁵ mitomycin-C-treated B-LCL as antigen-presenting cells or stimulators. For antigen-specific T cell assays, assays were performed in the presence or absence of either native antigen or immunogenic peptide. Protein antigens purified protein derivative (PPD), tetanus toxoid (TT), and hepatitis B surface antigen (HBsAg) were used at 10 µg/ml, as described (9, 17). Recombinant HBsAg was the gift of Merck, Sharp, & Dohme (West Point, PA). Synthetic peptides (MOMP 241–265, and TT 1274–1285) were used at indicated concentrations; the MOMP peptide was kindly provided by Miles Inc. (West Haven, CT). Assays of the MOMP-specific T cells included IL-2 at 30% of optimal levels for cell growth. T cell stimulation was measured by [3H]thymidine incorporation: (cpm in the presence of antigen – cpm in the absence of antigen) or, for allovreactivity (cpm in the presence of stimulators – cpm in the absence of stimulators). Data are median values from triplicate cultures from a representative experiment; SEM of relative responses (response to mutant/response to 8.1.6) were <15% in two or more replicate experiments.

**Antibodies and Antibody Binding Assays.** mAbs 16.23, 7.3.1.9, NDS-9, and CD6.B1 react with polymorphic HLA-DR determinants, as indicated on Table 1. mAbs L243 and Vl.15 react with monomorphic, conformational HLA-DR determinants, expressed on DR dimers. Exxon-shuffled Ezr/DRα mutants map the L243 determinant to the DRα chain (Chang, M.-D.Y., personal communication). mAb DA6.147 reacts with DRα, both as a monomer and when associated with DRβ. mAb B7/21.2 reacts with HLA-DR dimers and weakly with DPα and DPβ monomers. mAb DA6.231 reacts with HLA-DR and -DP dimers and -DR and -DP β chain monomers. Primary references for these antibodies are cited in reference 9, with the exceptions of NDS-9 (reference 25), L243 (reference 26), and DA6.231 (reference 27). Cell surface radiolabeling was performed as described (17). Immunofluorescence analysis was carried out using saturating amounts of unlabeled primary antibody, followed by fluoresceinated goat anti-mouse IgG (H+L) (Tago Inc., Burlingame, CA). Staining with secondary antibody alone was used as a control for background fluorescence. Samples were analyzed on an EPICS-C (Coulter Electronics, Inc., Hileah, Florida).

Sequence Analysis. Sequence analyses of the DRA and DRB genes of 8.1.6 and 10.24.6 were carried out as described (18). Briefly, cDNAs prepared from poly(A)+ RNA from 8.1.6 and 10.24.6 were subjected to PCR amplification using DRA and DRB primer pairs (see reference 18 for primer sequences). Size-selected double-stranded PCR DNA was subjected to dideox sequencing using
Reconstitution of HLA-DR Dimer Stability in SDS Using Added Ion Exchange-HPLC and Sequence Analysis of HLA-DR-associated Peptides.

For 16.23, 7.3.19, and VI.15, binding ratio: cpm bound by mutant - cpm bound by negative control/cpm bound by 8.1.6 - cpm bound by negative control. The negative control is 9.22.3, a DR-null mutant derived from 8.1.6 (reference 17). The binding ratios for mutants represent median values ± SEM from three or more independent assays. For NDS-9, CD6.B1, and L243, data are derived from immunofluorescence analysis. Binding ratio: corrected mean channel fluorescence (linear scale) of mutant/corrected mean channel fluorescence (linear scale) of 8.1.6. Data were corrected for background fluorescence assessed by binding of secondary antibody alone. The partial reconstitution of 16.23 epitope expression in the 10.24.6-DRA transfectant in all likelihood reflects continued expression of some mutant dimers in the transfectant. Some of the data on mutant 9.5.3 have been reported previously (9).

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Results

Mutant 10.24.6 Expresses an Altered DRα Chain. In an effort to isolate clones with single amino acid substitutions in the HLA-DR3 molecule, we chemically mutagenized progenitor 8.1.6, an HLA-DR/DQ hemizygous, DR3-expressing B-LCL, and selected mutant derivatives that had lost binding of a DR3-specific antibody, 16.23 (see Materials and Methods).
82 domains are shifted compared to the corresponding domains of HLA-carbohydrate addition site on a schematic representation of the HLA-A2 structure (Fig. 1, A and B). In addition, the mutation introduces a N-linked glycosylation sequence (Asn-X-Ser), which directs carbohydrate addition at residue 94N (Fig. 1, A and B). DRα chains in 10.24.6 are indeed modified by an extra N-linked carbohydrate moiety; they are ~3 kD higher in apparent molecular mass than DRα chains of progenitor 8.1.6 (Fig. 2, A and B) and this molecular weight disparity is abolished when DRα chains of 10.24.6 and 8.1.6 are treated with endoglycosidase F, which removes N-linked sugars (Fig. 2 A).

The Mutant HLA-DR Heterodimers Are Expressed at Normal Levels, but Have Altered Conformation. To determine the level of cell surface expression of the mutant HLA-DR3 molecules, we measured binding to 10.24.6 cells of a panel of DR3-reactive mAbs. 10.24.6 cells bind several antibodies at levels comparable to progenitor 8.1.6 (Table 1, antibodies CD6.81, VI.15), indicating normal or near normal surface abundance of the mutant DR3 dimers. The formation of HLA-DR α/β dimers in 10.24.6 implies integrity of the pairing interactions known to occur within the α1/β1 domain of class II dimers (31, 32) and argues against the possibility that substantial structural changes are propagated from the site of the α2 domain mutation. However, the HLA-DR3 molecules expressed by 10.24.6 have lost binding of some antibodies (Table 1; antibodies 16.23, 7.3.19, NDS-9) specific for polymorphic (DRB3) determinants that map to the peptide-binding (α1/β1) domain (33, 34), implying altered conformation in this domain. The altered binding of these antibodies is a consequence of the DRα mutation, as 10.24.6 cells transfected with a wild type DRA cDNA (10.24.6-DRA) regain expression of a relevant epitope (Table 1; mAb 16.23); the L243 (DRα) determinant is a predicted van der Waals contact; in the crystal structure of HLA-DR1, the relative positions of the α2 and β2 domains are shifted compared to the corresponding domains of HLA-A2, allowing contact between side chains of α96 and β118 (14). This α/β contact, which is likely conserved in HLA-DR3 molecules, may be disrupted by the 10.24.6 DRA mutation, however, α/β dimers are nonetheless formed (see text and Table 1).

Figure 1. (A) Locations of the 10.24.6 mutation and the resulting carbohydrate addition site on a schematic representation of the HLA-A2 structure. The structure of the MHC class I molecule, HLA-A2 (heavy chain and β2-microglobulin) is shown to approximate the structure of the folded HLA-DR molecule, which shares structural features with MHC class I molecules (13, 14). The transmembrane regions of DRα and β are not shown. Carbohydrate adduct is indicated as a branched chain, but is not drawn to scale. (α96...β118) is a predicted van der Waals contact; in the crystal structure of HLA-DR1, the relative positions of the α2 and β2 domains are shifted compared to the corresponding domains of HLA-A2, allowing contact between side chains of α96 and β118 (14). This α/β contact, which is likely conserved in HLA-DR3 molecules, may be disrupted by the 10.24.6 DRA mutation, however, α/β dimers are nonetheless formed (see text and Table 1). (B) Amino-acid sequence (single-letter code) of residues 92-98 of DRA from mutant 10.24.6 and progenitor 8.1.6. The 10.24.6 mutation (C→T) at codon 96 is shown. 8.1.6 sequence in this region is identical to published DRA sequence (47). N-linked glycosylation sequence is underlined.

10.24.6, DRA point mutant; mutants 9.5.3, 9.10.3, and 10.53.6, 8.1.6-derived C2P-1 presentation mutants (reference 9 and E. Mellins, unpublished data); 9.22.3, 8.1.6-derived DR-null mutant, which expresses HLA-DQ and HLA-DP molecules (17); 8.1.6, progenitor line. Unboiled, whole-cell extracts were analyzed by one-dimensional SDS-PAGE and immunoblotted with mAb DA6.147, which reacts with DRα monomers (left); or with anti-DR and -DP mAb DA6.231 (right). Because B7/21.2 reacts only weakly with monomers, the altered stability of the DP molecules in mutant 9.5.3 is best evaluated by the disappearance of DP dimer (lane 4, middle). DR-null mutant 9.22.3 is included as a control for antibody specificity; as expected, the dimer band from 9.22.3 is equivalent in intensity to that of 8.1.6 with the anti-DP antibody (middle) but of lower intensity than that of 8.1.6 with antibody that recognizes both DR and DP molecules (right). HLA-DR dimers of 10.24.6 appear unaffected by the 10.24.6 DRA mutation, as expected, since DRα/DPβ dimers are expressed infrequently, if at all, in these cells (E. Mellins, unpublished results).
minant is apparently also affected by the 10.24.6 mutation. Of interest, the pattern of binding of anti-DR3 antibodies to mutant 10.24.6 resembles that observed with the 8.1.6-derived C2P-1 mutants, which have wild type DRA and B gene sequences, but are deficient in intracellular formation of peptide/class II molecule complexes (e.g., mutant 9.5.3, Table 1). These results suggest that the DR molecules of 10.24.6 and those of C2P-1 mutants have similar changes in conformation at their peptide binding domains.

The Mutant HLA-DR Dimers Are Unstable in SDS Detergent To further assess the structure of class II molecules in mutant 10.24.6, we utilized an assay of class II dimer stability. Stability in SDS detergent solutions is a characteristic of a proportion of the mature class II molecules that have bound peptide (29, 35-37); class II dimers of C2P-1 mutants, on the other hand, are unstable in SDS (9). As shown in Fig. 2 B, the vast majority of HLA-DR dimers extracted from mutant 10.24.6 resemble those from C2P-1 mutants (e.g., 9.5.3, 9.10.3, 10.53.6) and dissociate into monomers in SDS-PAGE. However, the HLA-DP dimers of 10.24.6 and progenitor 8.1.6 maintain stability in SDS, unlike HLA-DP dimers in C2P-1 mutants (Fig. 2 B, middle and right). The SDS instability of the mutant DR molecules in 10.24.6 indicates that these molecules are altered in conformation compared to the majority of the HLA-DR molecules in the progenitor cell; the fact that SDS instability is limited to HLA-DR molecules in 10.24.6 implies that this phenotype results from mutation of the DRA gene.

We considered several mechanisms that might be the basis of the SDS unstable phenotype of HLA-DR dimers in mutant 10.24.6. SDS instability is observed in nascent class II molecules before dissociation of I chain (36), and in class II molecules (e.g., I-A^d) synthesized in the absence of invariant chain (38). However, neither lack of nor prolonged association with intact invariant chains explains the SDS instability of the HLA-DR molecules of 10.24.6, as the kinetics of I association and dissociation from the mutant DR molecules are normal (Amaya, M., and E. Mellins, data not shown). SDS instability is also a feature of class II molecules that lack immunogenic peptides, such as insect cell-derived HLA-DR1 molecules devoid of peptides (39), and class II molecules of C2P-1 mutants, which are defective in generating peptide/MHC class II complexes. To determine whether altered peptide loading is the basis of SDS instability in the HLA-DR molecules of 10.24.6, we first used antigen presentation to class II-restricted T cells to assess formation of class II/peptide complexes from endocytosed proteins. Fig. 3 a shows a comparison of T cell stimulation by 10.24.6 and progenitor 8.1.6. When provided with intact protein as antigen, the 10.24.6 mutant is unable to stimulate DR3-restricted T cells (Fig. 3 a), whereas a somatic cell hybrid between 10.24.6 and a homozygous DRA deletion mutant (10.24.6 x 9.22.3) maintains the mutant phenotype (Fig. 3 a).

Mutant HLA-DR Molecules in 10.24.6 Bind DR-restricted Peptides. It was possible that the mutant DR molecules in 10.24.6 were unable to participate in the normal process of peptide loading because their altered structure rendered them unable to bind peptides. To evaluate this possibility, we first tested the ability of 10.24.6 cells to stimulate DR-restricted T cells with peptide antigen. As shown in Fig. 3 b, HLA-DR molecules expressed by 10.24.6 present two DR-restricted peptides, one from the MOMP of C. trachomatis (241-265) (Barbosa, J., manuscript submitted for publication) and one from TT (1274-1285) (reference 40). These results indicate binding of immunogenic peptides to DR molecules of 10.24.6, however, they leave open the possibility that only a small fraction of 10.24.6 DR molecules, sufficient for T cell stimulation (41), is capable of binding peptide. We therefore utilized an assay in which peptide binding to the mutant molecules was detected by reconstitution of SDS-stable dimers (29, 30). In the absence of added peptide, DR3 molecules affinity purified from mutant 10.24.6 dissociate into monomers in SDS without boiling, whereas DR3 molecules affinity purified from progenitor 8.1.6 maintain the dimeric state in SDS, unless boiled (Fig. 4). After incubation at pH 4.5 with either of two DR3-restricted peptides (MOMP [251-265] or mycobacterial heat-shock protein 65 [3-13] [reference 42]), a majority of the affinity purified DR molecules from 10.24.6 forms SDS stable dimers, implying they have bound peptide (Fig. 4). These results, together with the peptide presentation assays, indicate that the structural changes and functional defects in the DR molecules of 10.24.6 are unlikely to derive from an intrinsic inability of the mutant molecules to bind immunogenic peptides.

HLA-DR Molecules in Mutant 10.24.6 and in C2P-1 Mutant 9.5.3 Are Associated with Invariant Chain Peptides. The SDS instability of HLA-DR3 molecules in mutant 10.24.6 thus appears to reflect a defect in the loading of these molecules with naturally processed peptides. To directly evaluate peptide occupancy of DR molecules in 10.24.6, we affinity purified HLA-DR<alpha>/beta dimers, extracted the associated peptides, and analyzed the peptides by ion exchange and reverse phase HPLC. Further, as the antibody binding profile, dimer instability, and antigen presentation defects of the HLA-DR3 molecules of 10.24.6 mimicked those of the DR3 molecules of C2P-1 presentation mutants, we compared the profiles of peptides eluted from the DR3 molecules of 10.24.6 to those eluted from 9.5.3, a representative C2P-1 mutant. Fig. 5 a shows the chromatograms of HLA-DR3-associated peptides from mutants 10.24.6, 9.5.3, and progenitor 8.1.6. Based on integration of the A_284 trace of these chromatograms, mutant and progenitor-derived DR3 yielded similar amounts of total peptides per mole of protein (70-85% of the starting amount). However, the profiles of peptides extracted from HLA-DR3 molecules of the two mutants contain a set of prominent peaks
Figure 3. (a) HLA class II-restricted presentation of endocytosed, native protein antigens by mutant 10.24.6, progenitor 8.1.6, hybrid 10.24.6 x 9.22.3, or transfectant 10.24.6-DRA as antigen-presenting cells. T cells are designated by antigen and restricting element and include four antigen-specific T cell clones (specific for PPD, TT, HBsAg), 2 alloreactive T cell clones (specific for DR3B1 [DR17] and DQ2), an alloreactive, anti-DP4 T cell line and a class II-restricted polyclonal T cell line specific for TT. The PPD-specific clones differ in fine specificity (18). The three TT-specific lines shown were derived from different donors. The stimulation of the TT-specific, class II-restricted T cell line by mutant 10.24.6 likely reflects primarily DQ- or DP-restricted presentation, as ~90% of the response to 10.24.6 was observed using the DR-null mutant 9.22.3 as antigen-presenting cells (E. Mellins, data not shown). (b) Proliferation of MOMP-specific and TT-specific DR-restricted T cells stimulated with immunogenic peptides, presented by mutant 10.24.6, DR-null mutant 9.22.3, or progenitor 8.1.6. MOMP 241-265 in association with DR3/31 stimulates a MOMP-specific T cell clone. The peptide corresponding to residues 1274-1285 of TT in association with DP9 stimulates a subset of T cells within the polyclonal, class II-restricted TT-specific T cell line; stimulation of this line with native TT is shown in a (TT/class II).

Figure 4. Mutant HLA-DR molecules bind antigenic peptide and regain SDS stability. HLA-DR molecules were affinity purified from metabolically-labeled (5-h pulse/18-h chase) progenitor 8.1.6 and mutant 10.24.6 cells. DR molecules were treated as indicated (see Materials and Methods for details) and analyzed by SDS-PAGE. The bands in the dimer region of the boiled 8.1.6 sample and the non-boiled and boiled 10.24.6 samples likely reflect the "floppy" dimer conformation, which runs at a higher apparent molecular weight in SDS-PAGE (29).

that are present at most in trace amounts in the eluate of the progenitor cells. We identified the peptides corresponding to these peaks by a combination of NH₂-terminal Edman degradation and mass spectrometry after further purification by reverse phase HPLC. As shown in Fig. 5 b, the DR3-associated peptides from the mutants derive from a common region of the invariant chain (residues 80-103). Using A₂₁₄ absorbance, we estimated that at least 60% of the total isolated peptides from both 10.24.6 and 9.5.3 was derived from Ii. None of the same peptides was detectable by reverse phase chromatography or mass spectrometry in the eluate from 8.1.6 cells (Cameron, P., unpublished results). The observation that HLA-DR3 molecules from mutants 10.24.6 and 9.5.3 are associated with the same Ii-derived peptides reveals the struc-
both mutants HLA-DR molecules fail to mature to appropriate species. Middle (10.24.6) and bottom (9.5.3) chromatograms represent the profile obtained from the HLA-DR hemizygous progenitor cells. Middle (10.24.6) and bottom (9.5.3) chromatograms represent the HLA-DR3-associated peptides from mutant cells. (b) Sequences of HLA-DR3-associated invariant chain peptides. The numbers above the sequences refer to the amino acid positions within the invariant chain; residues are numbered from NH2 terminus of p33 form of Ii.

Discussion

In mutant 10.24.6, striking alterations in the structure and antigen presentation function of the HLA-DR molecule are associated with a point mutation in the DRA gene. The phenotype of the mutant molecules can be specifically attributed to the DRA mutation, as transfection of 10.24.6 cells with a wild type DRA gene restores the wild type phenotype (Table 1 and Fig. 3). The mutation results in two structural changes in the DRα chain: (a) loss of a proline residue, 96P, which is highly conserved in murine and human class II α chains (43) and likely interacts with a conserved β chain residue (Fig. 1 a); and (b) addition of a bulky carbohydrate adduct to the α2 domain. The latter effect of the mutation may be more critical for the phenotype of the molecules because the particular amino acid substitution in DRα, serine for proline, is a relatively conservative change (44).

The key features of the DR molecules expressed by the 10.24.6 mutant, loss of expression of certain antibody epitopes, altered stability in SDS detergent solution, and deficient DR-restricted presentation of endocytosed protein, but not peptide, suggest a defect in formation of complexes between DR molecules and naturally processed peptides. In this report, we demonstrate that the mutant molecules are predominantly associated with peptides derived from Ii (residues 80-103), and are in this regard similar to DR molecules of antigen presentation mutants with defects in an MHC-linked gene(s) (Fig. 5, and references 30, 45). Fragments derived from the same region of invariant chain have also been found in association with several class II alleles in both human and murine wild type cells (1, 2, 46). The Ii-derived peptides may represent products of Ii proteolysis that reassociate with class II molecules (in competition with available immunogenic peptides) or they may be residual fragments of Ii that require removal to allow binding of immunogenic peptides. The DR eluates from both mutant 10.24.6 and the C2P-1 mutant 9.5.3 also contain non-Ii peptides, but these have not yet been characterized. Analysis of these non-Ii peptides may also shed light on the nature of the defect(s) in the two mutant cells.

Mutant 10.24.6 is unique in having a defined genetic lesion in DRA that yields HLA-DR molecules predominantly associated with Ii-derived peptides. Several mechanisms may be envisioned to account for this phenotype in 10.24.6 cells. (a) The mutant DR molecules have increased affinity for Ii-derived peptides. While this alternative cannot be excluded, carbohydrate addition to DRα is more likely to introduce steric inhibition of a critical interaction than to enhance affinity for an associated peptide. (b) The DRA mutation alters trafficking of DR molecules such that they do not encounter immunogenic peptides. The mutant molecules are known to arrive at a peripheral endocytic compartment where Ii proteolysis is accomplished; however, peptide loading may require transport to another compartment. This transport may be mediated by a non-Ii chaperone, which does not recognize the mutant DR molecules. Alternatively, transport of DR molecules may require homotypic aggregation, which is blocked in the mutant molecules. Homotypic interaction between HLA-DR1 molecules occurs during crystallization (reference 14), and the resulting dimer of dimers includes an interface near the region of DRα altered by aberrant glycosylation in 10.24.6. (c) The DR molecules in 10.24.6 reach the appropriate subcellular location but are unable to participate in the peptide-loading process. The mutant molecules are capable of binding and presenting extracellular peptides. However, physiologic formation HLA-DR/peptide complexes may involve an accessory molecule that either removes Ii fragments or loads immunogenic peptides. The altered DRα chain in 10.24.6 could hinder the binding or activity of such a molecule.

In several of the mechanisms proposed to account for the 10.24.6 phenotype, interaction between the HLA-DR molecule and a non-Ii accessory molecule is postulated. Antigen presentation mutants with defects in the MHC-linked C2P-1 gene provide evidence for such a non-Ii accessory molecule and indicate its critical function in the generation of peptide/class II molecule complexes. Indeed, as mutation of the C2P-1 gene and the DRA gene both generate HLA-DR mol-

Figure 5. (a) Ion exchange-HPLC profiles of HLA-DR3-associated peptides from mutants and wild type cell lines. Upper chromatogram (8.1.6) represents the profile obtained from the HLA-DR hemizygous progenitor cells. Middle (10.24.6) and bottom (9.5.3) chromatograms represent the HLA-DR3-associated peptides from mutant cells. (b) Sequences of HLA-DR3-associated invariant chain peptides. The numbers above the sequences refer to the amino acid positions within the invariant chain; residues are numbered from NH2 terminus of p33 form of Ii.

LPKPPKPVSKMRMATPLLQAL
LPKPPKPVSKMRMATPLLQALPM
LPKPPKPVSKMRMATPLLQALPMK
PKPPKPVSKMRMATPLLQALPMK
PKPPKPVSKMRMATPLLQALPMK
ecules with similar characteristics, it is attractive to speculate that direct association between class II molecules and the C2P-1 protein occurs during normal class II molecule biosynthesis and that the region of the class II α chain affected by the 10.24.6 mutation mediates this interaction.

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Address correspondence to Dr. Elizabeth Mellins, The Children's Hospital of Philadelphia, 34th Street and Civic Center Boulevard, Room 8138, Philadelphia, PA 19104.

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