STRUCTURAL REQUIREMENTS FOR PAIRING OF α AND β CHAINS IN HLA-DR AND HLA-DP MOLECULES

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The class II antigens of the human MHC are cell surface heterodimers composed of noncovalently linked 35-kD α chains and 29-kD β chains. These antigens are present on macrophages, B lymphocytes, and other APC (for review, see reference 1). Class II antigens are capable of binding immunogenic peptides and thereby serve as ligand for the TCR (2, 3). Recent experiments have demonstrated that class II proteins are also functional ligands for the T cell surface antigen CD4 (4–6).

Class II antigens display a very high degree of allelic polymorphism, presumably to enable the species to mount the widest possible range of immune responses. At the individual level, diversity in class II antigens is provided by the existence of three isotypic forms in man, named HLA-DR, HLA-DQ, and HLA-DP. These isotypes are characterized by nonpolymorphic sequences unique to each isotypic α and β chain. HLA-DR, the immunodominant class II isotype, has a nonpolymorphic α chain and a highly polymorphic β chain. Most of the allelic variation in DR β chains is concentrated in three regions located in the NH_2-terminal half of the molecule, termed the β1 domain. Extensive allelic variability is also located in the first domain of the DQα, DQβ, and DPβ chains (7, 8).

A third form of diversity is possible if the α chain of one isotype pairs with the β chain of another, creating a so-called mixed isotypic pair. Such pairings have not been demonstrated on APC in vivo using available monoclonal and polyclonal serological reagents. The pair DRα/DQβ has been found in particular transformed B cell lines that express high levels of the DR α chain (9). Modulation of the level of expression of DR α chains in B cell lines controlled the formation of the DRα/DQβ pair, in that only cells expressing high levels of DRα mRNA produced this mixed isotypic pair (10). Cell surface expression of mixed isotypic pairs was detected in cells transfected with different combinations of α and β chains, presumably because high levels of mRNA for each chain were produced in those cells (11).

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[1] *Abbreviations used in this paper:* LTR, long terminal repeat; MXH, mycophenolic acid, xanthine, and hypoxanthine; RSV, Rous sarcoma virus.

The murine counterpart of the DRα/DQβ pair, namely Eα/Aβ, was expressed at the surface of transfected cells (12, 13), and was detected biochemically on a particular B cell lymphoma (14). Only the Aβ chain from the H-2^d haplotype was able to pair with the Eα chain, and this permissive pairing was controlled by the first 50 amino acids of the Aβ chain (15). Expression of the mixed isotypic pair Eα/Aβ in cells cotransfected with isotypically matched chains occurred only if the mismatched chain was present in excess, suggesting an inefficient assembly of the mixed isotypic pair (16). It is not known whether the nonpermissive combinations of mixed isotypic pairs fail to be expressed because they do not assemble intracellularly, or because of a defect in transport to the cell surface.

In this report, we present the development of an efficient transient transfection system for the expression of mutated human class II cDNA molecules. It was possible to demonstrate the presence of DR and DP antigens both by cell surface staining and by metabolic labeling. Mixed isotypic pairs did not assemble efficiently and were not detected at the cell surface. Using a series of chimeric β chain cDNAs containing DR and DP sequences transfected into cells expressing either the DR or DP α chain, regions of β sequence that are important for isotypic pairing were identified. These regions are different for DR and DP, and suggest that there are important constraints on secondary and tertiary structure that determine the ability of a given β chain to form a stable heterodimer.

Materials and Methods

Tissue Culture. The murine fibroblastic cell line DAP-3 (17) was used throughout the experiments. Cells were maintained in DME, high glucose formulation (Hazleton Biologics, Inc., Lenexa, KS), supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes buffer, pH 7.4, and 10 μg/ml gentamicin. Medium for the selection of cells transfected with the bacterial *gpt* gene (see below) consisted of DME supplemented as above plus mycophenolic acid (6 μg/ml), xanthine (0.25 mg/ml), and hypoxanthine (15 μg/ml) (MXH)[1] (18).

Antibodies. The following mAbs were used: SG465, anti-human class II (19); SG520, anti-DR,DP β chain (20); SG171, anti-DR,DP β chain (21) (generously provided by Dr. S. Goyert, Cornell University Medical College); 1B5, anti-DR α chain (provided by Dr. J. Bodmer ICRF, London) (22); and P4H5, a hamster mAb to murine invariant chain (Dr. S. Cullen, personal communication). Rabbit antisera to synthetic peptides corresponding to the COOH termini of DR or DP β chains were prepared as described (23). Briefly, synthetic peptides were prepared corresponding to the COOH-terminal 10 and 11 residues of the DR1 or DP2 β chains, respectively. These peptides were coupled to keyhole limpet hemocyanin via an additional NH_2-terminal cysteine residue. The peptide-carrier conjugate (200 μg) was mixed with CFA and injected subcutaneously. The rabbits were boosted at 2, 3, and 13 wk by the injection of an additional 200 μg of antigen in IFA. Sera were obtained 2 wk after the last injection and tested by antipeptide ELISA.

Stable Transfections. DAP-3 cells (5×10^5/100-mm dish) were transfected using the calcium phosphate coprecipitation technique (24). 15 μg of the recombinant plasmids RSV.5 DRα or RSV.5 DPα were used for each transfection. These plasmids contain both the class II cDNA under the control of the Rous sarcoma virus long terminal repeat (RSV LTR), as well as the *Escherichia coli* guanyl phosphoribosyl transferase gene under the control of the SV40 early promoter. Their construction will be described elsewhere. 2 d after transfection, the cells were placed into MXH-containing medium. Surviving cells were cloned by limiting dilution. Individual clones were tested for expression of the α chain cDNA either by mRNA determination, or by the expression of dimeric class II molecules after transient transfection of class II β chains (see below).

Construction of a Replicating cDNA Expression Vector for Murine Cells. The eukaryotic expression vector RSV.3 contains the RSV LTR, a multiple cloning site from pUC12, and SV40-derived polyadenylation sequences plus pBR322 sequences for bacterial replication and ampicillin resistance (25). To facilitate later cloning, this vector was digested at the unique Hind III site at the 5′ end of the small polylinker region, repaired with Klenow, and a Cla I linker was added. The resultant plasmid was then linearized at the Bam HI site at the 3′ end of the SV40 termination sequences. This DNA was ligated to a 3.6-kb fragment derived by digesting the plasmid pSV5-neo with Bam HI (26). This fragment contains the entire polyoma virus early region, including the origin of replication and genes for the small, middle, and large T antigens. The resultant plasmid, termed R3CPy-II (Fig. 1), contains two unique sites for cDNA insertion: Cla I and Sal I. For most of the class II constructs (see below) this vector was digested with both of these enzymes to allow directed cloning.

Generation of Mutant and Chimeric Class II β Chains. Inspection of the cDNA sequences for the DR1 and DP2 β chains (27) revealed several positions where either a unique restriction site already existed at the same place in each cDNA, or could be created by one or two point mutations (Fig. 2). The mutations required to accomplish this are given in Table I. First, the cDNAs for the DR1 and DP2 β chains were removed from the original cloning vector (27) with the enzymes Sma I and Bam HI, and the Bam HI site was filled in with Klenow enzyme. The cDNAs were then cloned into a Klenow-repaired Sal I site in the vector Bluescript SK(−) (Stratagene, La Jolla, CA). Recombinant plasmids were analyzed for the orientation of the insert, and clones with the 5′ end of the cDNA nearest the Cla I site of the vector were identified. The cDNA with the vector-derived flanking Cla I and Xho I sites was removed with the enzymes Eco RI and Kpn I, and inserted into replicative form of M13 mp19 digested with the same enzymes.

Mutagenesis was performed on these recombinant phages using the Mutagene Kit (Bio-Rad Laboratories, Richmond, CA) as directed by the supplier. Synthetic oligonucleotide primers for the mutagenesis were 36 mers produced on a DNA synthesizer (380B; Applied Biosystems, Inc., Foster City, CA) and purified by PAGE. The presence of the desired mutations was determined by DNA sequencing using the chain termination method (28). Efficiency of the mutagenesis was typically 75–100%.

Replicative form DNA was generated from M13 clones with verified mutations. This was digested with Cla I and Xho I, and the resultant mutant class II cDNA was isolated from a low melting point agarose gel. This insert was then cloned into either the expression vector for functional analysis or Bluescript for further manipulation.

Chimeric β chain cDNAs were generated by digesting the wild-type or mutant class II β chain cDNA cloned into Bluescript with either Cla I or Xho I and the restriction endonuclease

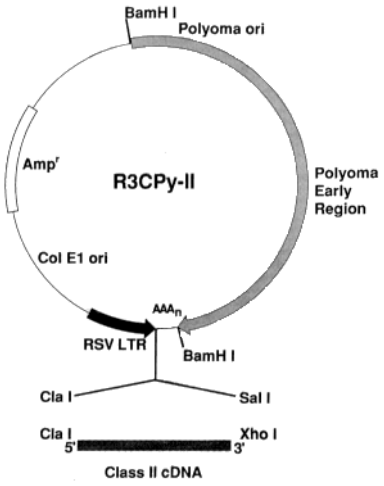

FIGURE 1. R3CPy-II, a polyoma-based eukaryotic expression vector. A vector for transient expression of cDNAs was constructed by ligation of a DNA segment containing the polyoma virus early region into the expression vector RSV.3 containing the ampicillin resistance gene, the bacterial origin of replication, the RSV LTR, and the SV40 poly(A) addition sequences (AAA_n). The direction of transcription of the early region genes, and the two unique cloning sites for cDNA insertion, are indicated.

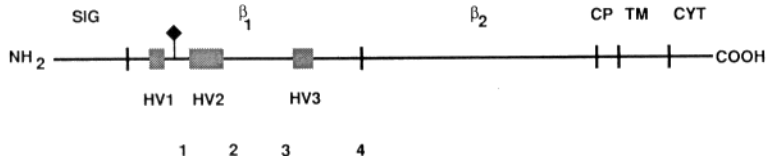

FIGURE 2. Position of mutations created in class II cDNAs. A schematic diagram of a human class II β chain is shown. The positions of the signal peptide (sig), β1 domain, β2 domain, connecting peptide (cp), transmembrane region (tm), and intra-cytoplasmic portion (cyt) are shown. HV1, -2, and -3 refer to the hypervariable regions of allelic diversity. The location of the single *N*-linked oligosaccharide is indicated by a diamond. The numbers 1, 2, and 4 refer to the positions of unique restriction endonuclease sites that were created by site-directed mutagenesis. Site 3 is the conserved Sca I site present in DR and DP β chain cDNA sequences. These four sites form the junctions for the chimeric cDNA molecules constructed between the DR and DP β chain sequences.

specific for the mutated site. DNA fragments containing 5′ sequences of one class II isotype were ligated to those coding for 3′ segments of the other isotype in the Bluescript vector. The resulting chimeric β chain cDNAs were then excised with Cla I and Xho I and cloned into the replicating expression vector. The authenticity of all constructs used for transfection was verified by restriction endonuclease digestion and DNA sequencing of the chimeric boundaries.

The chimera at amino acid 59 was constructed using the unique Sca I site common to all human class II β chain DNA sequences. Recombinant plasmids containing the wild-type DR or DP β chain cDNA in the Bluescript vector were digested with Sca I and Cla I to generate three fragments: a Cla I to Sca I fragment consisting of 5′ cDNA sequences, a Sca I to Sca I fragment containing 3′ cDNA sequences and vector sequences including one half of the ampicillin resistance gene, and an Sca I to Cla I fragment containing the remainder of the amp^r gene. The fragments were separated using low melting point agarose and the appro-

TABLE I

Mutations Required to Create Unique Restriction Sites

Site*	Enzyme	Recognition sequence	Isotype‡	Original sequence§			Mutated sequence‖		
				^{22} **Q**	**R**	**F**	-	-	-
1	Eco47 III	AGCGCT	DP	CAG	CGC	TTC	CAG	CGC	TTC
			DR	GAG	CG**G**	**G**TG	GAG	CG**C**	**T**TG
				E	**R**	**V**	-	-	**L**
				^{43} **D**	**V**		-	-	
2	Aat II	GACGTC	DP	GAC	GT**G**		GAC	GT**C**	
			DR	GAC	GT**G**		GAC	GT**C**	
				D	**V**		-	-	
				^{97} **P**	**R**		-	-	
4	Avr II	CCTAGG	DP	CCT	AGG		CCT	AGG	
			DR	CCT	AA**G**		CCT	A**G**G	
				P	**K**		-	**R**	

* Location of the unique restriction endonuclease site generated by site-directed mutagenesis, as shown in Fig. 2. Site 3 uses a unique Sca I site already present in DR and DP β chains.

‡ Class II β chain.

§ Sequences from DR1 and DP2 from reference 27. The derived amino acid sequence is shown above the DNA sequence. The nucleotide(s) that were mutated are shown in boldface. Superscript numbers indicate the position of the recognition sequence according to DRβ numbering.

‖ Amino acid changes as a result of mutagenesis are shown. A dash indicates no change.

priate DNA segments religated to generate the DR:DP and DP:DR chimeras. The chimeric cDNAs were put into the expression vector and the sequences verified as above.

The nomenclature of the chimeric β chains is as follows. The relative order of the two isotypes involved and the position of the first amino acid in the endonuclease recognition sequence is given (using the DR1 β chain numbering in reference 27). For example, RP22 is a β chain with an NH_2 terminus derived from DR1 and a COOH terminus derived from DP2, which is chimeric at the Eco 47 III site in the cDNA coding for amino acid 22.

Transient Transfections. The ability of chimeric class II β chains to pair with either DR or DP α chains was tested by transfecting their cDNA into fibroblasts stably expressing the α chain (above). 5×10^5 cells were plated in each T-25 tissue culture flask on the day before transfection. The next day, 5 μg of supercoiled plasmid DNA was diluted with 0.5 ml of serum-free DME for each flask. This was mixed with an equal volume of serum-free DME containing 1 mg/ml of DEAE-dextran (Pharmacia Fine Chemicals, Piscataway, NJ) and 100 μM chloroquine diphosphonate (Sigma Chemical Co., St. Louis, MO). The cells were washed twice with serum-free medium, and the DNA-containing solution (1 ml) was added to the cell monolayer. The cells were incubated with occasional rocking for 3 h in a humidified 37°C incubator. The DNA solution was then removed from the cells and replaced with complete medium for 24 h, then changed to complete medium supplemented with 10 mM sodium butyrate (Sigma Chemical Co.).

48 h after transfection, the cells were collected with trypsin/EDTA and incubated with saturating amounts of various mAbs on ice for 30 min. The cells were then washed and stained with fluoresceinated goat anti-mouse Ig. After the final wash, the cells were suspended in PBS containing 20 μg/ml propidium iodide (Calbiochem-Behring Corp., La Jolla, CA). Cells were analyzed on the FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA). A minimum of 10^4 live cells were analyzed.

Metabolic Labeling. Cells that had been transiently transfected with cDNAs for wild-type or chimeric class II β chains were methionine starved by a 1-h incubation in methionine-free MEM supplemented with 10% dialyzed heat-inactivated FCS. This medium was then replaced by medium containing 0.25 mCi of ^{35}S-methionine/ml (1 Ci = 3.67×10^{10} Bq). After 6 h at 37°, the cells were collected and washed extensively. They were then lysed in a buffer containing 0.5% NP-40, 0.15 M NaCl, 50 mM Tris/HCl, pH 7.5, 10 mM EDTA, 10 mM iodoacetamide, 10 mM L-methionine, and 1 mM PMSF. After 15 min on ice, the nuclei were spun out and the lysates precleared overnight at 4°C with 1% (vol/vol) normal rabbit or mouse serum and 10% (vol/vol) preswollen protein A-agarose. After microcentrifugation, the lysates were cleared a second time with 1:20 vol protein A-agarose. The cleared lysates were then immunoprecipitated with a combination of 1:20 vol of mAb and 1:20 volume of protein A-agarose for 2 h at 4°C. The precipitates were washed twice with lysis buffer made 0.5 M with NaCl, twice with lysis buffer made 0.25 M with NaCl, and once with lysis buffer alone. The pellets were solubilized by heating in 2% SDS and subjected to electrophoresis in 12% polyacrylamide gels under nonreducing conditions (29). The gels were fixed, soaked in En^{3}Hance (Dupont-New England Nuclear, Pittsburgh, PA), dried, and exposed to Kodak X-omat film at −70°. The M_r was determined using ^{14}C-methylated protein molecular weight standards (Amersham Corp., Arlington Heights, IL).

Results

Expression of Human Class II cDNAs in a Polyoma-based Replicating Vector. To rapidly analyze wild-type and mutant class II molecules, the vector R3CPy-II was constructed (Fig. 1). This vector contains sequences for bacterial replication and ampicillin resistance from pBR322, the RSV LTR to direct expression of inserted cDNAs, SV40 termination sequences, and the entire polyoma virus early region. Full-length, expressible cDNAs for the class II α and β chains were cloned into this vector. Murine fibroblasts were transfected with these recombinant plasmids and analyzed by flow microcytofluorimetry 48 h later. Initially, L cells were transfected with replicating plasmids for both the class II α and β chains simultaneously (not shown). However,

it was determined that higher levels of class II expression could be obtained if either the α or β chain was stably transfected and the partner chain transiently transfected. Using this technique, 30–50% of the cells express class II molecules on the cell surface (see below).

Expression of Matched, but not Mixed Isotypic Class II Molecules. L cells that had been stably transfected with either the DR or DP α chain were then transiently transfected with the DR and DP β chains encoded by the polyoma-based vector (Fig. 3). Only the matched isotypic pairs (DRα/DRβ and DPα/DPβ; Fig. 3, *A* and *D*, respectively) are expressed at the cell surface as detected by the antibody SG465, which reacts with all native class II molecules (19). The mixed isotypic pairs (DRα/DPβ and DPα/DRβ; Fig. 3, *B* and *C*, respectively) were not detected. These transfectants were also stained with L243 (anti-HLA-DR), B7-21 (anti-HLA-DP), and SG171 and SG520 (both anti-class II β chain). In all cases, only transfections of matched isotypic pairs were detected by these antibodies (not shown). In addition, transfections of the HLA-DQ α and β chain cDNAs along with DR or DP subunit cDNAs did not result in detectable cell surface class II expression, whereas the DQα/DQβ combination was easily detected with SG465 (not shown).

Intracellular Pairing of Mixed Isotypic Class II Molecules Is Inefficient. It was possible that mixed isotypic pairs were being formed, but that they were either not being transported to the cell surface, or that the antibody SG465 required the presence of a conformational determinant present only on normal pairs. To investigate these possibilities, cells stably expressing the DR or DP α chains were transfected with either the DR or DP β chain cDNAs, and class II proteins were biosynthetically radiolabeled with ^{35}S-methionine, detergent solubilized, and immunoprecipitated with rabbit antisera directed to distinct COOH-terminal β chain protein sequences. The immunoprecipitates were then analyzed by nonreducing SDS-PAGE. As shown in Fig. 4, a significant amount of either the DR or DP β chain is synthesized in all cells transiently transfected with replicating expression vectors (Fig. 4, lanes *C–F*). However, only in the cells containing the homologous isotypic pairs DRα/DRβ (Fig. 4, lane *C*) or DPα/DPβ (Fig. 4, lane *F*) does a significant amount of the α chain coprecipitate with the β chain. As shown in Fig. 4, lane *A*, the murine class II–associated invariant chain migrates just below the α chain in this electrophoretic system.

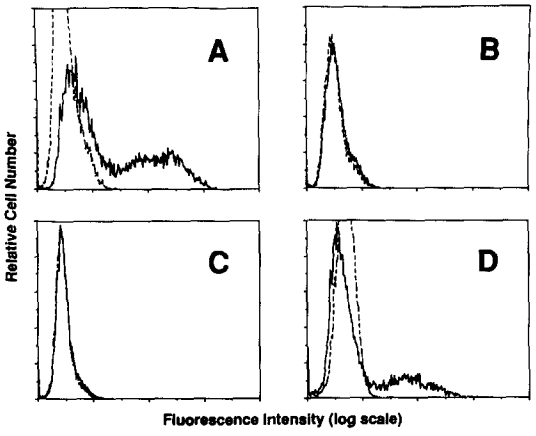

FIGURE 3. Transient transfection of murine fibroblasts with human class II cDNAs. L cells stably transfected with either the DR (*A* and *B*) or DP (*C* and *D*) α chain cDNA were transiently transfected with replicating expression vectors containing the DR1 β chain cDNA (*A* and *C*) or the DP2 β chain cDNA (*B* and *D*). Cells were stained with mAb SG465 48 h after transfection and analyzed by microcytofluorimetry.

It is intensely labeled due to a high methionine content. The invariant chain is only associated with paired α/β dimers. It is not coprecipitated with either the α chain (Fig. 4, lane *B*) or β chain alone (Fig. 4, lanes *D* and *E*). The band migrating ahead of the invariant chain corresponds to the expected position of p25, a proteolytic fragment of the invariant chain (30).

Expression of Chimeric DR/DP β Chains with DR and DP α Chains. To ascertain the important β chain sequences necessary for pairing with the matched α chain, chimeric β chain cDNAs were constructed. These constructs created β chains that had both DR and DP sequences. The positions of the crossover from one isotypic sequence to the other is shown in Fig. 2. In cases where the mutation needed to create the unique restriction site for chimera construction caused an amino acid change, an additional construct was made containing only that mutation within the wild-type DR or DP sequence. This β chain was tested along with the chimeras for association with both DR and DP α chains.

Fig. 5 shows the results of transient transfections of class II β chains containing DR and DP sequences chimeric at the junction between the β1 and β2 domains (amino acid 97) into murine fibroblasts stably expressing the DR or DP α chains. The β chain containing DR β1 and DP β2 sequences (RP97) was able to pair efficiently

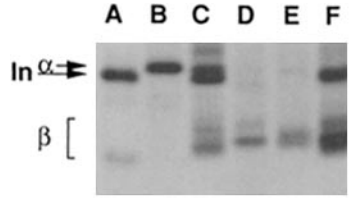

FIGURE 4. Metabolic labeling of class II molecules in transiently transfected L cells. Cells stably expressing the DR α chain (lanes *A–D*) or DP α chain (lanes *E* and *F*) were transiently transfected with the replicating vector without a cDNA insert (lanes *A* and *B*), with the DR β chain cDNA (lanes *C* and *E*), or with the DP β chain cDNA (lanes *D* and *F*). 48 h after transfection, the cells were labeled with ^{35}S-methionine. Immunoprecipitates of cell lysates using P4H5 (antimurine invariant chain, lane *A*), 1B5 (anti-DR α chain, lane *B*), or rabbit antisera directed against the COOH terminus of the DR β chain (lanes *C* and *E*) or DP β chain (lanes *D* and *F*) were analyzed by nonreducing SDS-PAGE. The relevant portion of a 12% polyacrylamide gel is shown. The position of the class II α and β chains and the class II-associated invariant chain (In) are indicated. The band migrating ahead of the invariant chain in lane *A* is a proteolytic fragment, p25 (30).

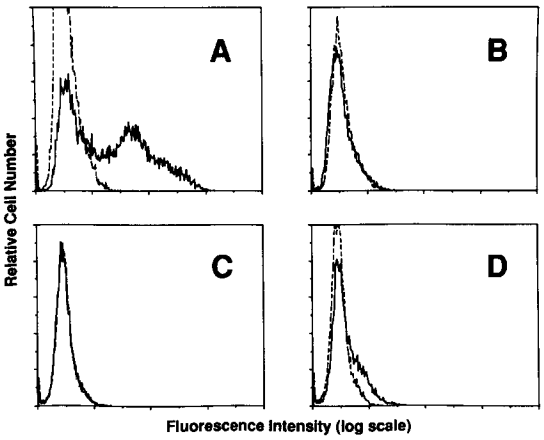

FIGURE 5. Cell surface expression of class II molecules with chimeric β chains. Cells expressing the DR α chain (*A* and *B*) or DP α chain (*C* and *D*) were transiently transfected with replicating vectors containing chimeric β chain cDNAs. The cDNA used in *A* and *C* contains the β1 domain of DR and the β2 domain of DP (RP97); that used in *B* and *D* has the β1 domain of DP and the β2 domain of DR (PR97). The expression of class II was determined by staining the cells with antibody SG465 and microcytofluorimetric analysis.

and be expressed at the cell surface (as judged by staining with SG465) only with the DR α chain. Conversely, a β chain having DP β1 and DR β2 sequences (PR97) would only pair with the DP α chain, although the level of expression per cell was lower than that seen in the DRα/RP97β combination. Thus, the presence of isotypically matched β1 sequences alone was sufficient to allow pairing.

The results of transfection of β chain cDNAs chimeric between DR and DP sequences at amino acids 22, 43, and 59 into DR and DP α chain-containing cells are summarized in Table II. Staining the cell surface with an anti-human class II reagent demonstrated that the DR α chain would pair and be expressed with β chains having DR sequence up to amino acid 22, as well as chimeras having DP sequence in their NH_2 termini up to positions 43 and 59. In addition to pairing with the PR97 β chain, the DP α chain was able to pair and express with the PR43 and PR59 β chains (which also paired with DRα). The PR22, RP43, and RP59 β chains were unable to pair and express with either the DR or DP α chain, as judged by cell surface staining with anti-DR (L243), anti-DP (B7-21), or anti-β chain (SG171 and SG570) mAb (data not shown).

Metabolic Labeling of Human Class II Molecules Containing Chimeric (DR/DP) β Chains. As before, the apparent lack of expression of certain chimeric β chains with DR or DP α chains could be explained simply by either a lack of the appropriate mAb epitope, or by lack of surface expression of intracellular α/β dimers. To address both of these issues, cells stably expressing the DR or DP α chains were transfected with wild-type or chimeric β chains, labeled with ^{35}S-methionine, and lysed, and the lysates were immunoprecipitated with rabbit anti-COOH-terminal sera as above. The immunoprecipitates were separated on nonreducing polyacrylamide gels (Fig. 6).

TABLE II

Transfection of β Chain cDNAs Chimeric between DR and DP Sequences into DR and DP α-containing Cells

Chimeric β chain*	DRα		DPα	
	Percent‡	Level§	Percent	Level
RP22	40	14	0	-
RP43	0	-	0	-
RP59	0	-	0	-
RP97	54	13	0	-
PR22	0	-	0	-
PR43	41	35	55	73
PR59	48	4	29	29
PR97	0	-	22	3

* L cells expressing either the DR or DP α chain were transiently transfected with class II β chains that were chimeric for DR and DP sequences (see text for details). After 48 h, the cells were analyzed for surface expression of human class II by microcytofluorimetry.

‡ Percentage of cells binding the mAb SG465.

§ Level of fluorescence intensity determined by the ratio of the mean fluorescence intensity of the SG465$^+$ cell population to the mean fluorescence intensity of the cells stained with goat anti-mouse IgG alone. Representative values for the transient expression of the unmutated isotypes are 30-50% positive cells at a level 22 times background for DR, and 30-35% positive cells at a level 22 times background for DP.

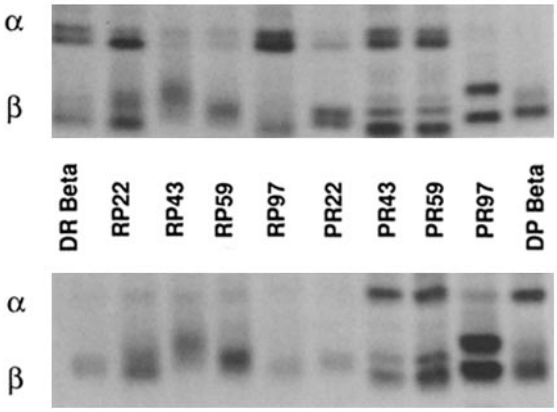

FIGURE 6. Metabolic labeling of cells transfected with chimeric class II β chain cDNAs. L cells stably transfected with the DR α chain (*A*) or DP α chain (*B*) were transiently transfected with the indicated chimeric β chain cDNAs. Metabolically radiolabeled proteins were immunoprecipitated with rabbit antisera specific for the COOH terminus of the chimeric β chain (DR or DP) and analyzed as in Fig. 4. The position of the class II α and β chains are indicated.

The surface staining of cells with the antibody SG465 correlated with the presence of significant intracellular pairing of the α and β chains. Those combinations that were not detected at the cell surface resulted in little or no α chain coprecipitated with the β chain. Therefore, the lack of expression is due to poor initial α/β pairing, rather than a defect in transport of intracellular dimers to the cell surface, or a lack of antibody binding.

With one exception, the biosynthetically labeled β chain chimeras migrate as poorly defined bands of heterogeneous mobility. The PR97 β chain migrates as a distinct doublet. The variation in the pattern of β chain migration from one chimera to another is highly reproducible (Fig. 6, *A* and *B*). It is not known whether this variability in mobility represents differences in intra-chain disulfide bond formation, differences in post-translational processing, or merely the effect of different amino acid compositions.

Discussion

At present, the exact nature of the structure of class II molecules is unknown. The three-dimensional structure can be inferred by comparison to a class I molecule for which a crystal structure has been obtained (31). The alignment of sequences and preservation of amino acid residues essential for tertiary structure (e.g., disulfide bonds and salt bridges) provides presumptive evidence for the schematized structure shown in Fig. 7. Such modeling alone cannot predict whether there will be isotypic differences in the exact three-dimensional structure of class II, nor whether the lack of mixed isotypic pair expression at the cell surface is due to intrinsic incompatibilities between the α and β chains of different isotypes.

The experiments described in this report were designed to answer some of these questions. Specifically, whether mixed isotypic pairs of human class II molecules form intracellularly, whether they are expressed at the cell surface, and which parts of the class II β chains are important for the ability to pair with α chains. To address these questions efficiently, a polyoma-based transient transfection system was developed. The expression vector uses the RSV LTR as a promoter for the inserted cDNA. This promoter has been shown to be very active in a variety of eukaryotic cells (32). In particular, it has been used previously in stable transfections of L cells with human

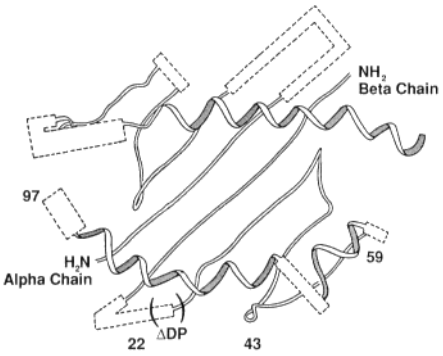

FIGURE 7. Model of the class II antigen-binding site. A model of the three-dimensional structure of the α1 and β1 domains of class II is shown (adapted from reference 31 with permission of the authors). The numbers indicate the approximate positions of the sites used to create chimeric class II β chains between DR and DP sequences. Amino acid 22 is in the region between the first two β strands, 43 is between the third and fourth β strands, 59 is in the beginning of the α helix, and 97 is at the beginning of the β2 domain. The parentheses mark the region of the β chain where the DP sequence has a two-amino acid deletion compared with DR (ΔDP).

class II α and β chains (25). The polyoma virus early region in the vector, R3CPy-II, contains the polyoma origin of replication as well as genomic sequences for the three early proteins, large, middle, and small T antigens. Large T antigen is necessary for viral (and vector) replication and is responsible for immortalization of cells (33). Middle T antigen is necessary for the induction of the transformed phenotype (34). Small T antigen serves to enhance the level of viral replication (35). Although the presence of middle and small T antigens is not absolutely necessary for polyoma replication, they were included in the expression vector in an attempt to maximize the potential amount of class II mRNA present in the cell. A similar vector containing the polyoma virus early region was previously used for expression of genomic clones encoding Ig H chains (36).

In our transient system, all three isotypes of human class II can be individually expressed at the surface of murine fibroblasts. They form the appropriate heterodimers intracellularly, and bind to the murine invariant chain during biosynthesis. They each retain reactivity for specific and broadly reactive anti-class II mAbs. Thus, these class II molecules appear to be identical to the class II molecules synthesized in human APC. The demonstration of inefficient mixed isotypic pairing is unlikely to be due to the experimental system, even though minor species- or tissue-specific differences in post-translational modifications, such as carbohydrate processing, cannot be ruled out. Expression of a mixed isotypic pair, such as DRα/DQβ, probably requires high intracellular levels of these chains, together with the availability of a chain present in excess over its isotypic partner (10). Such mixed isotypic molecules are probably rare and are unlikely to contribute functionally to class II antigen diversity.

The lack of expression of mixed isotypic molecules in the transient transfection system made it possible to test which regions of class II β chains were important for pairing with α chains. A series of chimeric β chains was constructed between DR and DP β chain sequences. The expression pattern of these chimeras with the DR or DP α chains leads to two immediate conclusions. First, that HLA-DR and HLA-DP are structurally different insofar as their requirements for α/β pairing. For example, the chimeric β chain RP22, having only the first 22 amino acids of the DR β chain sequence, is sufficient to pair with the DR α chain. The PR22 β chain does not pair with the DP α chain. The DP α chain will pair efficiently with a chimeric β chain having DP sequences for the first 43 amino acids (PR43). The

converse β chain chimera, RP43, does not pair with the DR α chain. Similar results are found with β chain chimeras centered around amino acid 59.

The second conclusion is that since the pairing of α and β chains of different isotypes cannot be accounted for simply on the basis of primary sequence, then secondary and tertiary structural considerations must play an important role. The model of the class II antigen-binding site developed by Brown et al. (31) (Fig. 7) can serve as a useful model for the interpretation of the data in this report. The pairing of the RP22 β chain with the DR α chain implies an interaction between the two β strands in the center of the floor of the antigen-binding site. In the case of the DP β chain, additional β sheet structure (amino acids 1-43) is needed to pair efficiently with the DP α chain. The pairing of the PR43 and PR59 β chains with both the DR and DP α chains, and the lack of pairing of the RP43 and RP59 β chains with either α chain, have several possible explanations. First, there is a two-amino acid deletion in the DP β chain sequence relative to the DR sequence just after amino acid 22. Although the DR and DP α chains are of identical length, the tertiary structure of the DP α chain may have evolved to compensate for this deletion. In addition, the pairing pattern of these chimeras may demonstrate the effects of different regions of α/β interaction. The failure of RP43 and RP59 β chains to assemble with α chains may reflect specific interactions between the α helix and the underlying β strands in DP β chains. Further, the pairing of the PR43 and PR59 β chains with the DR α chain may imply that there is significant interaction between the α-helical portions of the β1 domain (amino acids 59–97) of DR and portions of the DR α chain. Studies with α chain chimeras will provide additional insight on this point.

A different structure for the DR and DP NH_2-terminal domains would have important implications in class II antigen function. For example, the nature of immunogenic peptides that bind to DP may be different than those that bind to DR. In this way, different isotypes of class II would serve to broaden the immune response of an individual by presenting a wider array of antigens. In addition, there may be differences in the way class II molecules interact with the TCR, or with coreceptor molecules such as CD4. Finally, there have been several recent reports that bacterial toxins that serve as "super antigens," activating T cells in vitro, bind directly to class II molecules (37, 38). There is a definite hierarchy of binding to different class II isotypes among the various toxins tested. The molecular basis for these differences should be readily testable using class II molecules with chimeric and point-mutated α and β chains.

Summary

To test for the assembly of human MHC class II molecules having an α chain from one isotype (HLA-DR, -DQ, or -DP) and the β chain of another (mixed isotypic pairs), murine fibroblasts were transfected with expressible cDNAs encoding the different class II α and β chains. A rapid and efficient transient transfection system was developed using a polyoma virus–based vector. Typically, 30–50% of cells transfected using this system expressed high levels of class II molecules on their surface, but only with matched isotypic pairs. Biochemical analysis of cells transfected with matched or mixed isotypic pairs of the DR and DP molecules revealed that only matched chains could pair efficiently inside the cell. Thus, the lack of expression

of the two mixed isotypic pairs is due to inefficient primary assembly of the class II molecule and not to a processing or transport defect.

To define what region of the β chains controlled their assembly with α chains, a series of chimeric cDNA molecules containing both DR and DP β chain sequences were constructed. Expression of these chimeric β chains with DR and DP α chains was determined by cytofluorimetry and biochemical analysis. Both α chains paired with β chains in which only the β1 domain was isotypically matched. In contrast, the pattern of expression of chimeras made at other points within the β1 domain was different for DR and DP. These data show that different areas of primary sequence are important for the assembly of different human class II isotypes, and suggest that HLA-DR and -DP molecules have different secondary or tertiary structures in their NH_2-terminal domains.

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References

1. Kaufman, J. F., C. Auffray, A. J. Korman, D. A. Schackleford, and J. L. Strominger. 1984. The class II molecules of the human and murine major histocompatibility complex. *Cell.* 36:1.
2. Unanue, E. R., and P. M. Allen. 1987. The basis for immunoregulatory role of macrophages and other accessory cells. *Science (Wash. DC).* 236:551.
3. Sette, A., S. Buus, S. Colon, J. A. Smith, C. Miles, and H. M. Grey. 1987. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature (Lond.).* 328:395.
4. Gay, D., P. Maddon, R. Sekaly, M. A. Talle, M. Godfrey, E. Long, G. Goldstein, L. Chess, R. Axel, J. Kappler, and P. Marrack. 1987. Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature (Lond.).* 328:626.
5. Sleckman, B. P., A. Peterson, W. K. Jones, J. A. Foran, J. G. Greenstein, B. Seed, and S. J. Burakoff. 1987. Expression and function of CD4 in a murine T-cell hybridoma. *Nature (Lond.).* 328:351.
6. Lamarre, D., D. J. Capon, D. R. Karp, T. Gregory, E. O. Long, and R.-P. Sékaly. 1989. Class II MHC molecules and the HIV gp120 envelope protein interact with functionally distinct regions of the CD4 molecule. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3271.
7. Trowsdale, J., J. A. Young, A. P. Kelly, P. J. Austin, S. Carson, H. Meunier, A. So, H. A. Erlich, R. S. Spielman, J. Bodmer, and W. F. Bodmer. 1985. Structure, sequence, and polymorphism in the HLA-D region. *Immunol. Rev.* 85:5.
8. Korman, A. J., M. Boss, T. Spies, R. Sorrentino, K. Okada, and J. L. Strominger. 1985. Genetic complexity and expression of human histocompatibility antigens. *Immunol. Rev.* 85:45.
9. Lotteau, V., L. Teyton, D. Burroughs, and D. Charron. 1987. A novel HLA class II molecule (DRα-DQβ) created by mismatched isotype pairing. *Nature (Lond.).* 329:339.
10. Lotteau, V., J. Sands, L. Teyton, P. Turmel, D. Charron, and J. L. Strominger. 1989. Modulation of HLA class II antigen expression by transfection of sense and antisense DRα cDNA. *J. Exp. Med.* 169:351.
11. Long, E. O., S. Rosen-Bronson, S. Jacobson, and R. P. Sekaly. 1990. Isotypic diversity and function of HLA class II molecules. *In* Molecular Biology of HLA Class II Antigens. J. Silver, editor. CRC Press. Boca Raton, FL. In press.

12. Germain, R. N., and H. Quill. 1986. Unexpected expression of a unique mixed-isotype class II MHC molecule by transfected cells. *Nature (Lond.).* 320:72.
13. Malissen, B., N. Shastri, M. Pierres, and L. Hood. 1986. Cotransfer of the $E\alpha^d$ and $A\beta^d$ genes into L cells results in the surface expression of a functional mixed-isotype Ia molecule. *Proc. Natl. Acad. Sci. USA.* 83:3958.
14. Spencer, J. S., and R. T. Kubo. 1989. Mixed isotype class II antigen expression: a novel class II molecule is expressed on a murine B cell lymphoma. 1989. *J. Exp. Med.* 169:625.
15. Sant, A. J., N. S. Braunstein, and R. N. Germain. 1987. Predominant role of amino-terminal sequences dictating efficiency of class II major histocompatibility complex $\alpha\beta$ dimer expression. *Proc. Natl. Acad. Sci. USA.* 84:8065.
16. Sant, A. J.,and R. N. Germain. 1989. Intracellular competition for component chains determines class II MHC cell surface phenotype. *Cell.* 57:797.
17. Margulies, D. H., G. H. Evans, K. Ozato, R. D. Camerini-Otero, K. Tanaka, E. Appela, and J. G. Seidman. 1983. Expression of H-2D^d and H-2L^d mouse major histocompatibility antigen genes in L cells after DNA-mediated gene transfer. *J. Immunol.* 130:463.
18. Mulligan, R. C., and P. Berg. 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA.* 78:2072.
19. Goyert, S. M., and J. Silver. 1983. Further characterization of HLA-DS molecules; implications for studies assessing the role of human Ia molecules in cell interactions and disease susceptibility. *Proc. Natl. Acad. Sci. USA.* 80:5719.
20. Shaw, S., A. Ziegler, and R. DeMars. 1985. Specificity of monoclonal antibodies directed against human and murine class II histocompatibility antigens as analyzed by binding to HLA-deletion mutant cell lines. *Hum. Immunol.* 12:191.
21. Goyert, S. M., J. E. Shively, and J. Silver. 1982. Biochemical characterization of a second family of human Ia molecules, HLA-DS, equivalent to murine I-A subregion molecules. *J. Exp. Med.* 156:550.
22. Adams, T. E., J. G. Bodmer, and W. F. Bodmer. 1983. Production and characterization of monoclonal antibodies recognizing the chain subunits of human Ia alloantigens. *Immunology.* 50:613.
23. Maloy, W. L., J. E. Coligan, Y. Barra, and G. Jay. 1984. Detection of a secreted form of the murine H-2 class I antigen with an antibody against its predicted carboxyl terminus. *Proc. Natl. Acad. Sci. USA.* 81:1216.
24. Graham, F. L., and A. J. Van Der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456.
25. Jacobson, S., R. P. Sekaly, C. L. Jacobson, H. F. McFarland, and E. O. Long. 1989. HLA class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. *J. Virol.* 63:1756.
26. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327.
27. Tonnelle, C., R. DeMars, and E. O. Long. 1985. DOβ: a new β chain gene in HLA-D with distinct regulation of expression. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2389.
28. Sanger, F., S. Nickler, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 22:680.
30. Thomas, L. J., Q. V. Nguyen, W. L. Elliot, and R. E. Humphreys. 1988. Proteolytic cleavage of I_i to p25. *J. Immunol.* 140:2670.
31. Brown, J. H., T. Jardetzky, M. A. Saper, B. Samraoui, P. J. Bjorkman, and D. C. Wiley. 1988. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature (Lond.).* 332:845.

32. Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. USA.* 79:6777.
33. Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin. 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. *Nature (Lond.).* 300:713.
34. Treisman, R., U. Novak, J. Favaloro, and R. Kamen. 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. *Nature (Lond.).* 292:595.
35. Cherington, V., B. Morgan, B. M. Spiegelman, and T. M. Roberts. 1986. Recombinant retroviruses that transduce individual polyoma tumor antigens: effects on growth and differentiation. *Proc. Natl. Acad. Sci. USA.* 83:4307.
36. Deans, R. J., K. A. Denis, A. Taylor, and R. Wall. 1984. Expression of an immunoglobulin heavy chain gene transfected into lymphocytes. *Proc. Natl. Acad. Sci. USA.* 81:1292.
37. Mollick, J. A., R. G. Cook, and R. R. Rich. 1989. Class II MHC molecules are specific receptors for staphylococcus enterotoxin A. *Science (Wash. DC).* 244:817.
38. Scholl, P., A. Diez, W. Mourad, J. Parsonnet, R. S. Geha, and T. Chatila. 1989. Toxic shock syndrome toxin 1 binds to major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA.* 86:4210.