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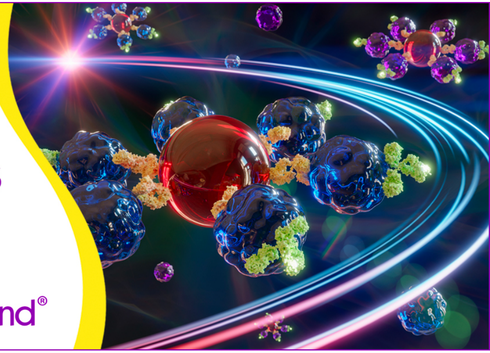

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TRANSFER OF POLYMORPHIC MONOCLONAL ANTIBODY EPITOPES TO THE FIRST AND SECOND DOMAINS OF HLA-DR β-CHAINS BY SITE-DIRECTED MUTAGENESIS[1]

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We used site-directed mutagenesis of HLA-DR β-chains to localize the binding sites for two polymorphic DR-binding mAb to residues in the first and second external domains, respectively. Transfer of three first domain α-helical residues, G73, R74 and N77, normally present in DR3a and DRw52a, to a DR4 β-chain was sufficient for recognition of this mutant DR molecule by a DR3-specific mAb, NDS 9. A polymorphism controlling the binding of a DR4-specific mAb, GS 359-13F10, was mapped to a tyrosine at position 96 of the DR4 β-chain second domain by the construction of a chimeric DR molecule containing a DR2-first domain and DR4-second domain. The mapping of these two polymorphic epitopes to specific positions on the DR β-chain will allow further structural and functional analysis of the DR molecule.

HLA class II molecules, DR, DQ, and DP, are cell surface heterodimeric glycoproteins encoded by linked genes in the HLA D-region and constitutively expressed on a limited number of cell types. These molecules are characterized by extensive intraspecies allelic polymorphism. They function by binding fragments of processed Ag thereby creating determinants that can be recognized by self-class II-restricted T lymphocytes. Class II molecules are also the targets of strong allogeneic responses by T and B cells, responses that form the basis for identification of class II specificities. Certain class II specificities are associated with increased susceptibility to autoimmune diseases. Therefore, it is important to elucidate the role of specific polymorphisms in the function of individual class II alleles.

The class II DR molecule differs from other class II molecules in important respects. The α-chain gene of DR (DR-A) is nonpolymorphic which greatly simplifies the interpretation of the contribution of DR β-chain polymorphisms to DR function. However, the number of active DR β-chain genes (DR-B genes) varies from one DR haplotype to the next so that individual haplotypes may code for more than one form of DR simultaneously expressed on the cell surface. An example of this is the DR3 haplotype of the DRw52 group (1), where the more polymorphic DR-B1 locus product defines some DR3-associated serologic and T cell specificities and the less polymorphic DR-B3 locus product defines additional specificities. This complication is compounded by the patchwork nature of polymorphism within DR-B genes. For example, the DR3-B1 gene is the product of a gene conversion event and contains sequence elements donated by a co-expressed DR-B3 allele that is linked to DR3-B1 in some DR3 haplotypes, but not in others (2). In such instances, the analysis of the role of individual amino acid polymorphisms in the function of a given DR allele can be greatly simplified by using the twofold approach of DNA-transfected cells and site-directed mutations.

We derived several human B cell transfectants expressing mutated DR molecules to locate serologic epitopes on the DR β-chain. We investigated the contribution to the DR3 serologic specificity of three polymorphic residues acquired in the DR3-B1 conversion event by mutational transfer of these same residues to the DR4 molecule. These three residues, when transferred to the first domain α-helical region of DR4, were sufficient for the recognition of this mutant by a DR3-specific mAb. We also investigated the role of polymorphic second domain residues in defining the DR4 supertypic specificity by constructing a DR2-first domain/DR4-second domain chimeric molecule. This construct was recognized by a DR4-specific mAb presumably by binding to DR4-specific residues in the second domain. Based on these and previous results of Alber et al. (3), we identified a tyrosine residue at position 97 of the DR4 β-chain second domain as critical for binding. These results indicate that second domain polymorphisms as well as first domain polymorphisms contribute to the formation of epitopes recognized by mAb.

MATERIALS AND METHODS

cDNA expression. A new cDNA expression vector was constructed from the plasmids pHEBO (4) and pCMVβ (5). pCMVβ was digested with *NotI* to remove the *Escherichia coli* β-galactosidase gene and the vector portion was closed by ligation. Digestion of this vector with *ScaI* and *SalI* released a 2.0-kb fragment containing the human cyotmegalovirus immediate early promoter/enhancer, the splice donor and acceptor sequence of SV40 16S and 19S late mRNAs, a unique *NotI* site, the SV40 late polyadenylation signal, and a portion of the β-lactamase gene of pUC19. This fragment was isolated by agarose gel electrophoresis and purified (Gene-clean, Bio 101, La Jolla, CA). A 5.7-kb *ScaI SalI* restriction fragment derived from pHEBO and carrying the EBV *oriP*, the hygromycin resistance gene

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of *E.coli*, and the remaining part of the β-lactamase gene was similarly purified and was ligated to the pCMV-derived fragment to generate pBEx1 (Fig. 1*A*). *NotI* fragments containing full length cDNA of HLA DR-B (1.2 kb) or HLA DR-A (917 bp) were subcloned into the *NotI* site of pBEx1 (Fig. 1*A*) and were screened for proper orientation by PCR.

Site-directed mutagenesis of DR β-chains. Directed mutations were generated by modification of previously described methods (6, 7). Figure 1*B* shows the general scheme used to introduce amino acid substitutions into DR β-chains. Sense and antisense oligonucleotide primer pairs hybridizing within the coding sequence were synthesized to effect the desired amino acid alterations. The same primers also encoded additional nucleotide substitutions needed to create an artificial restriction site (e.g., *SalI*) by silent mutation. Other primers hybridizing to the 5′ and 3′ untranslated regions of the DR-B cDNA were paired with the mutagenic primers in two separate PCR reactions to amplify the gene in 5′ and 3′ parts. After removal of primers (Centricon, Danvers, MA), the PCR products were phenol-extracted and ethanol-precipitated. Purified PCR products were digested with appropriate restriction enzymes, combined in equimolar ratios and ligated into the pBEx1 expression vector in a three-point ligation. In addition to the artificial restriction site introduced in the coding region by PCR reaction, this fully forced subcloning used a conserved *SacI* site located in the signal peptide of all DR-B genes and a *HindIII* site located in the 3′ untranslated region of many DR-B genes. For some constructions, a version of the pBEx1 expression vector was used in which the *HindIII* site had been mutated to a *BstEII* site by the same general approach used for coding region mutagenesis.

Insertion of artificial restriction sites within the coding region often permits further mutations to be introduced on double-stranded oligonucleotides synthesized to generate the appropriate cohesive ends after hybridization. A DR-B gene with multiple restriction enzyme cassettes bracketing polymorphic regions can be obtained by multiple rounds of PCR mutagenesis.

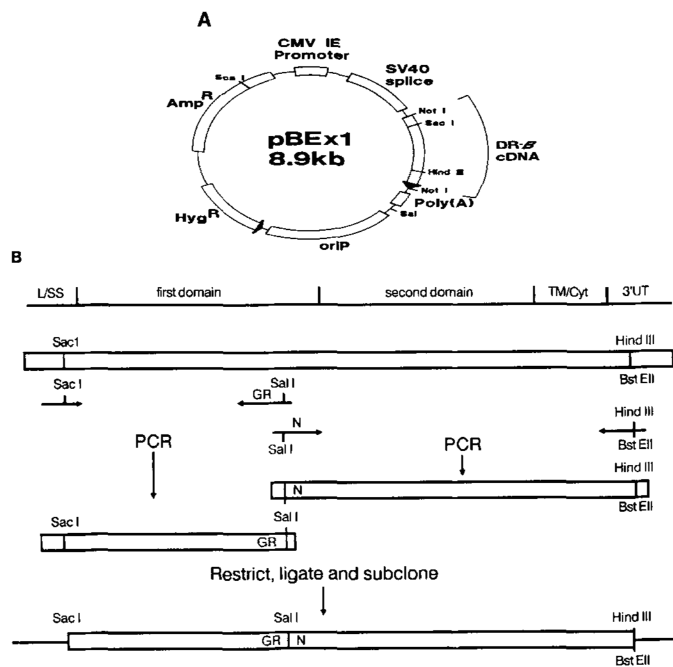

Figure 1. cDNA expression and mutagenesis. *A*, pBEx1 expression vector. Shown is the pBEx1 DR-B cDNA expression vector. pBEx1 contains the EBV origin of replication *oriP* permitting the plasmid to replicate episomally in EBV-transformed B lymphoblasts (4) and the hygromycin resistance gene *hph* (*Hyg*^R) for drug selection in B lymphoblasts. DR-A or DR-B cDNA subcloned into a unique *NotI* site is transcribed from the strong human cytomegalovirus immediate early promoter/enhancer. pBEx1 DR-B cDNA vector features a DR-B cDNA cassette defined by a unique *SacI* site in exon 1 (signal peptide) of all DR β-chain genes and either a unique *HindIII* site located in exon 6 (3′ untranslated region) of most DR β-chain genes or a *BstEII* site artificially introduced at the same location. *Amp*^R, β-lactamase gene of *Escherichia coli; SV40 splice*, RNA splice donor and acceptor sequence; *Poly(A)*, SV40 late polyadenylation sequence. *B*, Mutagenesis of DR-B cDNA by polymerase chain reaction (PCR). Schematic representation of DR-B cDNA. Synthetic oligonucleotides (*horizontal arrows*) incorporating specific nucleotide substitutions to mutate coding sequence and to add an artificial restriction site (*SalI*) by silent mutation. Oligo primer pairs (*arrowheads*, 5′ or 3′ orientation) are used to generate two PCR products corresponding to 5′ or 3′ portions of the cDNA. PCR products are restricted, joined at the site of mutation by *SalI* cohesive ends and joined to the vector by unique *SacI* and *HindIII* or *BstEII* sites.

HLA-DR cDNA clones. DR-A and DR-B cDNA were derived by specific reverse transcription and PCR amplification of total RNA prepared from homozygous cell lines (Tenth International Histocompatibility Workshop). The HLA-DR cDNA clones used for transfection and mutagenesis were as follows. The DR3-B1 locus product representing the DR3a gene (DRB1*0301) was derived from the line QBL (Tenth International Workshop no. 9020). The DR4-B1 locus product representing the DR4a gene (DRB1*0401) was derived from the line JBUSH (Tenth International Workshop no. 9035). The DR6-B1 locus product representing the DR6a gene (DRB1*1301) was from the line HHKB (Tenth International Workshop no. 9065) as reported (2). The DR2-B1 locus product (DRB1*0201) was derived from the cell line AMAI (Tenth International Workshop no. 9010).

The DR6-B3 locus product DR52a (DRB3*0101) was from the line HHKB (Tenth International Workshop no. 9065) as reported (2). The DR12-B3 locus product DR52b (DRB3*0201) was from the line BM16 (Tenth International Workshop no. 9038). The DR6-B3 locus product DR52c (DRB3*0301) was from the line WT46 as reported (8). DR4x52a refers to the construct where certain amino acid residues found on DR52a were transferred to DR4. The use of a slash, for example DR2/4, refers to a hybrid molecule where the first domain was derived from the gene designated before the slash and the second domain was derived from the gene designated after the slash. Sequencing was performed by chain termination (9) on all cDNA generated by amplification to verify construction and to rule out PCR-produced errors.

Transfection. An HLA class II⁻ variant of the human Burkitt's lymphoma cell line P3HR1 (10) was provided by Dr. Robert Humphreys, University of Massachusetts Medical School, Worcester, MA. This P3HR1 variant had no detectable surface expression of HLA-DR. No DR-α- or -β-chain mRNA was observed using a sensitive PCR technique (11), and transfections of either DR-A or DR-B cDNA expression vectors alone failed to restore cell surface DR expression. P3HR1 was maintained in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT).

DR-A and DR-B cDNA clones in pBEx1 were cotransfected into P3HR1 cells by electroporation (Progenetor II, Hoefer Scientific Instruments, San Francisco, CA). Cells (10^7) harvested from log-phase growth were resuspended in 0.5 ml serum-free OptiMEM (GIBCO BRL, Gaithersburg, MD) containing 100 μg of each supercoiled plasmid. After 10 min incubation on ice in disposable cuvettes (Hoefer), cells were electroporated at 260 V, 1350 μF using a cuvette electrode. After an additional 10 min incubation on ice, cells were diluted 10-fold in RPMI 1640 with 10% FCS and were cultured for 36 h. Transfected cells were subsequently selected for by culture in the same medium supplemented with 800 μg/ml Hygromycin B (Sigma Chemical Co., St. Louis, MO). After 7 to 14 days of selection for Hygromycin B-resistant cells, maximal cell surface DR expression was obtained, and uncloned, bulk transfectant cultures were used for all subsequent mAb analyses.

Immunofluorescence staining and flow microfluorimetry analysis. Indirect immunofluorescence was performed using HLA-DR-specific mAb. Murine mAb NDS 9, reactive with cells expressing DR3 (12), was provided by Dr. Susan Fuggle, John Radcliffe Hospital, Oxford, U.K. Rat mAb GS 359-13F10, reactive with cells expressing DR4 (3) was provided by Dr. Susan Radka, Oncogene, Seattle, WA. Monomorphic anti-HLA DR mAb L243 (13) was obtained from the American Type Culture Collection, Rockville, MD. DR transfectants in P3HR1 (5×10^5 cells) were incubated with saturating amounts of mAb for 20 min at 4°C. The cells were washed three times with 2.5 ml PBS with 1% BSA and 0.02% sodium azide and then incubated for an additional 20 min with affinity-purified, fluorescein-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG or goat anti-rat IgG (Organon Teknika, West Chester, PA) as required. After a second washing step, the cells were fixed in suspension with 1% paraformaldehyde in PBS. Flow cytometric analyses of 5×10^3 cells were performed using a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA) and data analysis programs purchased from Becton Dickinson.

RESULTS

The gene encoding the DR3/Dw3 specificity (DR3a) was generated by a gene conversion event (2). In this intra chromosomal gene conversion, sequence from the DR-B3 locus (DRw52a) was donated to the DR-B1 locus (DRw6a).

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Transfer of sequence results in substitution of seven amino acids (Fig. 2), including most notably, the gain of two positively charged residues, K71 and R74, and the loss of two negatively charged residues, D70 and E71. The net effect of this transfer is the acquisition of new serologic and T cell specificities by the converted DR molecule. Most of the amino acids transferred by this event are predicted to lie in the α-helical region of the DR β-1 domain where their side chains can influence the binding of antibodies and T cell recognition (15). We hypothesized that the mutational transfer of the α-helical amino acid substitutions from this region to a serologically unrelated DR molecule such as DR4 might impose some DR3-like specificities on a mutated DR molecule. By the technique described in Figure 1*B*, we generated a mutant DR4 gene in which residues A73, A74, and T77 of DR4a were replaced with G73, R74, and N77, the corresponding amino acids from DRw52a. The complete first domain sequence of this mutant, named DR4x52a, is shown in Figure 2, *line 2*.

cDNA clones of DR3a, DR4a, DRw52a, and the DR4x52a mutant were inserted into the expression vector pBEx1 (Fig. 1*A*) designed for high-level expression in EBV-transformed B cells. Transfectants were produced by co-electroporation of DR-B genes with a pBEx1 DR-A cDNA vector into the class II$^-$ B cell line P3HR1 (9). After selection against untransfected parental cells, Hygromycin B-resistant cells were analyzed for the binding of anti-DR mAb by fluorescent flow cytometry. Figure 3 shows the results of one such analysis.

We used the monomorphic anti-DR mAb L243 to quantitate overall DR expression by individual transfectants. L243 binding (DR) reveals only minor variation in the level of DR expression among the transfectants. mAb NDS 9 has been shown to react strongly with all DR3$^+$ cells and weakly with certain other cells including all cells of the DRw52 supertypic group (11). As expected, NDS 9 binds to the DR3a transfectant (Fig. 3*A*) and also to the DRw52a transfectant (Fig. 3*B*) but does not bind to the DR4 transfectant (Fig. 3*C*). NDS 9 also fails to bind to transfectants expressing DRw52b, DRw52c, or several other unrelated DR-B genes (data not shown), suggesting that the reactivity of this polymorphic mAb is controlled by residues donated in the aforementioned gene conversion. NDS 9 binds to the DR4x52a mutant (Fig. 3*D*) at a level comparable to that of the DR3a and DRw52a transfectants, suggesting that transfer of residues G73, R74, and N77 is sufficient for recognition of a DR4 molecule by NDS 9. These same residues must therefore form part of the NDS 9 epitope.

mAb GS 359-13F10 reacts with all cells expressing the DR4 specificity (3). Inasmuch as we transferred residues associated with DR52a and DR3a to a DR4 molecule, we were interested in what effect these mutations might have on the binding of this polymorphic mAb. 13F10 binds to the DR4 transfectant (Fig. 3*C*) although failing to bind to the DR3 and DRw52a transfectants (Fig. 3, *A* and *B*). 13F10 also binds to the DR4x52a transfectant even though three amino acid substitutions have been made in the first domain α-helical region of this mutant (Fig. 3*D*). This result suggested that residues controlling the reactivity of 13F10 may lie outside this α-helical region.

Comparison of second domain sequences for DR-B alleles reveals shared amino acid polymorphisms that closely correlate with supertypic serologic specificities (16). We sought to determine if residues in the second domain of DR4 β-chains control a public epitope that is recognized by 13F10. We constructed chimeric DR β-chains by introducing an artifical *Hin*dIII site before position 88 of a DR4/Dw4 gene by mutation of codon 87 from GAG to GAA. The breakpoint at position 88 between the amino-terminal and carboxyl-terminal portions of the chimeric β-chains does not correspond precisely with the boundary between first and second domains at residues 94 and 95. Even so, all residues from position 88 through position 95 are nonpolymorphic among DR β-chains, making chimeric β-chains joined at this position functionally equivalent to first domain/second domain exchanges.

We made two constructs involving the exchange of first and second domains. In one construct, DR4 second domain sequence was joined to the serologically unrelated DR2 (DRB1*0201) first domain sequence (DR2/4). A second construct joined the DR2-B1 first domain sequence to a DR1 second domain sequence (DR2/1) to serve as a control. Transfectants of DR2/4 and DR2/1 chimeric β-chains and a new DR4a transfectant were produced in P3HR1 and were analyzed for the ability to bind mAb 13F10 (Fig. 4). The binding of monomorphic mAb L243 reveals some variation in DR expression among the transfectants. As expected, mAb 13F10 binds to the DR4 transfectant (Fig. 4*A*). It also binds to the transfectant expressing the DR2/4 chimeric β-chain at a level consistent with lower overall DR expression by this transfectant (Fig. 4*B*). mAb 13F10 fails to bind the transfectant expressing the DR2/1 chimeric β-chain (Fig. 4*C*) suggesting that binding of 13F10 to the DR2/4 transfectant is not controlled by residues present on the DR2 first domain.

The reactivity of 13F10 with a DR molecule containing a DR2/4 chimeric β-chain localizes the residues controlling the binding of this mAb to the DR4 β-chain second domain. Only two residues are unique to DR4 B1 alleles within the second domain sequence (16), Y96 and L181. No other residues within the less polymorphic second domain shared among DR4-B1 alleles are not also present in other DR alleles not reactive with 13F10. The DR2/4 chimeric β-chain molecule, shown schematically in Figure 5*A*, can be compared with a DR molecule containing a half domain shuffled DR β-chain produced by Alber et

```
          6   10        20        30        40        50        60        70        80        90
cons      RFLE--KSECHFFNGTERVRFLDRYFHNQEEYVRFDSDVGEYRAVTELGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVVESFTVQRR
DR4a     (----QV-H------------------YH-------------------------------------K--------------G--------)
DR4aX52a (----QV-H------------------YH-------------------------------------K-GR--N--------G--------)
DR3a     (----YST-------------Y----------N---------F-----------------------K-GR--N-----------------)
DRw52a   (----LR--------------Y----------FL------------------V--S----------K-GR--N--------G--------)
DRw52c   (----LL----------------E--------F-------------------V--S----------K-GQ--N-----------------)
DRw52b   (----LL----------------E-H-------A------------R-------------------K-GQ--N-----------------)
DRw6a    (----YST------------------------N---------F-------------------I--DE-----------------------)
```

Figure 2. First domain amino acid sequence comparison of normal and mutant DR beta chain alleles: DR4a and DR4x52a, involved in mutant construction; DR3a, DRw52a, and DRw6a, involved in an intrachromosomal gene conversion event; DRw52c and DRw52b, other allelic genes at the DR-B3 locus. Sequences described in the References 2, 8, and 14.

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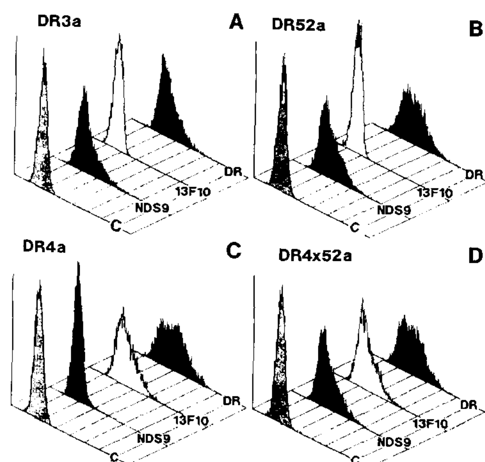

Figure 3. Fluorescence histograms of P3HR1 DR transfectants stained with mAb NDS 9 (anti-DR3, DRw52a), 13F10 (anti-DR4), or L243 (anti-DR monomorphic). *A*, DR3a transfectant. *B*, DRw52a transfectant. *C*, DR4a transfectant. *D*, DR4x52a transfectant. (*C*), background staining with fluorescenated second step reagent alone. (*DR*), L243 staining.

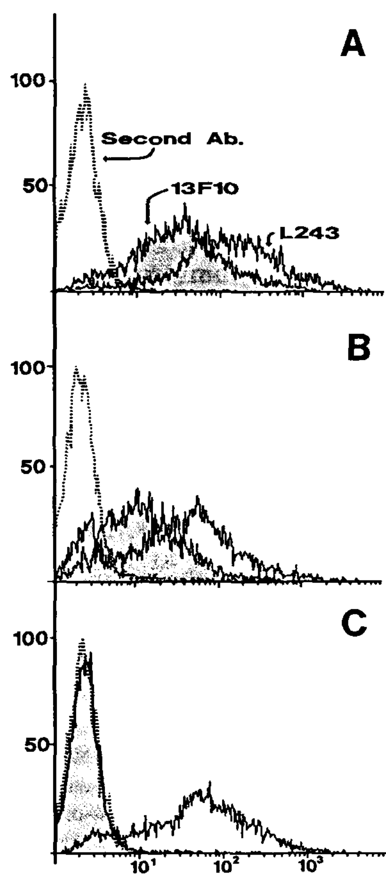

Figure 4. Fluorescence histograms of P3HR1 DR transfectants stained with mAb 13F10 and L243. *A*, DR4a transfectant. *B*, DR2/4 chimeric transfectant. *C*, DR2/1 chimeric transfectant. *Dotted histogram*, background staining with fluorescenated second step reagent alone. *Shaded histogram*, 13F10 staining. *Unshaded histogram*, L243 staining.

al. (3) (Fig. 5*B*). This construct localized the epitope for mAb 13F10 to a region encoded between a *Taq*I site in codon 40 and an *Oxa*NI site in codon 97 of the mature DR4/Dw13 β-chain. The region of overlap between these two constructs, both of which bind 13F10, is defined by residues 95 to 97 (Fig. 5*C*). DR4-specific residue Y96 is present within the overlap region, whereas L181 is not. L181 is replaced by V181 in the half domain-shuffled DR β-chain of Alber et al. (3). Therefore, L181 is not required for the binding of 13F10. In addition to Y96, residues V95 and P97 are present within the overlap region. Inasmuch as V95 and P97 are completely non-polymorphic within DR β-chains, the polymorphism Y96 must control the binding of 13F10 to DR4 and must form part of the 13F10 epitope on the DR4 second domain.

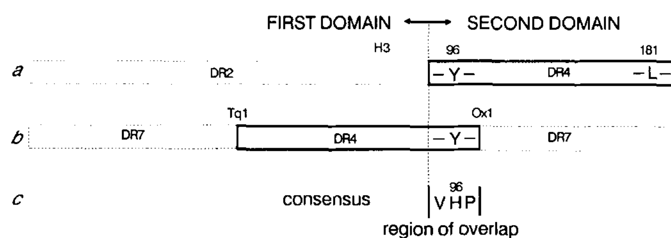

Figure 5. Region of overlap between DR4 cDNA mutants reactive with mAb 13F10. *a*, Schematic representation of DR2/4 chimeric beta chain showing unique residues Y96 and L181 in the DR4 derived second domain. *b*, Representation of a half domain shuffled DR 7/4/7 β-chain mutant constructed by Alber et al. (3) including DR4 residues from a *Taq*I site in codon 40 to an *Oxa*NI site in codon 97. *c*, Overlap region of DR consensus sequence shared by both mutants.

DISCUSSION

Allelic products of the DR-B3 locus of the DRw52 group of HLA haplotypes demonstrate important functional polymorphisms despite lower cell surface expression (17) and limited sequence diversity (2) when compared with alleles at the DR-B1 locus. DRw52 allelic products control at least two serologic specificities designated TR22 (18) and TR81 (19). DRw52 alleles are recognized by alloreactive and Ag-specific T cells (13). Some immune responses of allergen-specific T cells isolated from atopic individuals appear to be controlled by DRw52 alleles (20). The presence of the DRw52a allele is highly associated with the production of pathogenic anti-PL-A1 antibodies by PL-A1⁻ mothers in the clinically important syndrome neonatal alloimmune thrombocytopenia (21). These results highlight the important role of DRw52 polymorphisms in controlling immune responses in normal and disease states.

As a first step in determining the contribution of individual amino acid polymorphisms to the function of DRw52 alleles, we transferred three polymorphic amino acids found in DRw52a and DR3a β-chain alleles to a DR4a molecule. Transfer of these three amino acids, G73, R74, and N77, was sufficient for recognition of the mutant DR4x52a molecule by a DR3- and DRw52a-specific mAb, NDS 9. Inasmuch as the reactivity of mAb NDS 9 is closely correlated with the serologic specificity, TR 81, present on the DR-B1 locus product of DR3 and the 52a allelic product of the DR-B3 locus (22), it is plausable that these three amino acids transfer the TR 81 allospecificity to DR4a as well.

Our finding that substitution of only a few amino acids is sufficient to transfer a polymorphic mAb epitope on HLA-DR is similar to previous studies of IA and HLA-DQ. Landais et al. (23) showed that introduction of the $A^k\alpha$-specific residue E75 into $A^b\alpha$ was sufficient for reactivity with a polymorphic mAb. Buerstedde et al. (24) demonstrated gain of $A^d\beta$-specific mAb reactivities with substitution of one or a few residues on exposed turns or α-helical positions of $A^k\beta$. Kwok et al. (25) showed that an E for G substitution at position 45 of the DQ 3.2 β-chain

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results in both gain and loss of polymorphic mAb reactivities.

Although three amino acids are sufficient for transfer of NDS 9 reactivity, the "footprint" of this mAb may include additional residues (Fig. 6A). It is also possible that some of the transferred residues are not required for reactivity. In addition to the three residues transferred as part of the mutation of DR4a, three other polymorphic positions are common to all NDS 9-reactive β-chains and are shown in Figure 6A in *lower case*. These three residues, L67, Q70, and K71, already present on DR4a (Fig. 2, *line 1*), are not by themselves sufficient for NDS 9 reactivity. L67 and K71 have side chains predicted to point into the Ag binding site (15), and thus are not positioned favorably for direct contact with Ab. Indirect interaction via a common peptide occupying the binding site seems unlikely, because the β-sheet regions involved in peptide binding differ dramatically in all three NDS 9-reactive β-chains. In contrast to L67 and K71, Q70 is postulated to point upward and into the top part of the site and thus to be positioned more favorably for interaction with an Ab.

Of the three residues transferred by mutation (Fig. 6A, *upper case*), R74 is a critical amino acid for determining NDS 9 binding. This stems from the observation that NDS 9 does not bind to DRw52b or 52c, whose helical regions are identical to those of the NDS 9-reactive β-chains except at position 74 (Fig. 2). Using an immunoselected mutant of DR3, Mellins et al. (26) have recently shown that responses by all of seven DR3-restricted or allospecific T cell lines tested depended on the presence of arginine at position 74. These results further support the critical role of R74 for the function of the DR3 allele.

The other two amino acids, G73 and N77, are postulated to point outward and upward, respectively, and thus to be favorably positioned for Ab contact. Although we cannot exclude any of these residues from participation in NDS 9 binding, we predict that the NDS 9 "footprint" will require R74 and additional residues, perhaps Q70 and N77, on adjacent α-helical turns. Nonpolymorphic residues may also contribute to NDS 9 binding by maintaining epitope conformation or by directly contacting the antibody combining site.

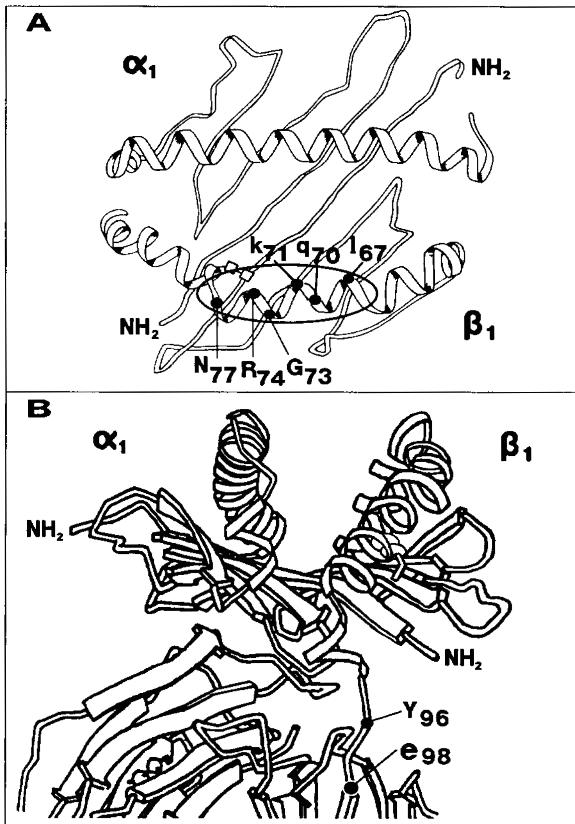

Figure 6. Model of the HLA-DR molecule based on homology to the HLA class I structure (27). *A*, Amino acids controlling mAb NDS 9 binding are indicated on the DR β-chain first domain α-helical region within a putative Ab epitope. G73, R74, and N77 (*upper case*), amino acids mutationally transferred to DR4a. L67, Q70, and K71 (*lower case*), polymorphic residues present in DR4a. *B*, The position of Y96 controlling the binding of mAb 13F10 binding on the DR β-chain second domain. E98 is indicated in lower case to denote its presence in all 13F10-binding DR β-chain constructs.

A tyrosine residue critical for binding a second polymorphic mAb, GS359-13F10, was localized to the DR4 β-chain second domain, specifically to position 96. Second domain location of the epitope for this DR4-specific mAb supports our contention that supertypic Ab will often recognize public epitopes in the second domain (16). Based on half domain shuffling experiments, it has been suggested previously (3) that 13F10 may bind to the carboxyl-terminal portion (α-helical region) of the DR4 β-chain first domain. However, these results are reconciled by comparison of the structural details of DR4 constructs positive with 13F10. All of the exon shuffled constructs of Alber et al. (3) also contain three amino acids derived from the second domain (Fig. 5, *line b*). Within the region of overlap among DR4 β-chain constructs that are 13F10-reactive, only three amino acids are shared. Of these three, two are nonpolymorphic among DR β-chains. Only Y96 is a polymorphic residue shared among 13F10-reactive constructs, and Y96 is only found in DR4-B1 chains. It is worth pointing out that all 13F10 reactive constructs in the present study and in that of Alber et al. (3) also have the polymorphism E98 in the second domain. E98 may also be necessary for the 13F10 epitope, but E98 is not by itself sufficient, as E98-containing DR7-B1 chains fail to react with 13F10 (3). Non-polymorphic residues flanking Y96 and E98 in the second and first domains may also be necessary but not sufficient for the 13F10 epitope.

Using the HLA class I crystal structure (27) as a model of the DR molecule, it is possible to position Y96 and E98 on the predicted DR structure (Fig. 6B). Y96 is located on a short descending loop of three amino acids that connects the distal end of the β-chain α-helical region with the first β-strand of the β-chain second domain Ig fold, whereas E98 forms the first residue of this β-strand. This region is most probably fully accessible to Ab contact. Although positioned barely on the second domain, the location of the 13F10 epitope does identify a unique "face" of the DR molecule. Localization of the 13F10 epitope on the DR β-chain second domain and of the NDS 9 epitope on the first domain α-helical region will make these mAb valuable reagents for the study of DR-ligand interactions.

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