Cell Surface Expression and Function of an HLA Class II Molecule With Class I Domain Configuration

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Summary

Recombinant major histocompatibility complex (MHC) class II molecules were expressed with extracellular polypeptide domains reorganized to form heavy (H) and light (L) chains (\(\alpha_2\beta_1\beta_2\) and \(\alpha_3\)) analogous to class I. Accurate protein folding and dimerization is demonstrated by the ability of this 3 + 1-DR1 construct to bind class II-restricted peptides and stimulate CD4+ T cells. Cell surface expression of a functional class II molecule consisting of H and L chains supports the validity of current class II models and affirms the evolutionary relatedness of class I/II. MHC functions that differ between class I/II may be influenced by domain configuration, and the use of domain-shifted constructs will allow examination of this possibility.

Materials and Methods

cDNA Clones. Full-length HLA-DR \(\alpha\) and \(\beta\) chain cDNA clones in the pCD expression vector were used in these studies. The DRA cDNA, encoding the DR \(\alpha\) chain, was isolated as described (8). The DRB1*0101 cDNA, encoding the DR[\(\beta^1\)*0101] (formerly DR1/31) chain (9), was obtained from the American Type Culture Collection ([ATCC] Rockville, MD).

Splicing of cDNA Encoding \(\alpha\) and \(\beta\) Chains. The PCR method of splicing by overlap extension (10) was used to generate cDNAs encoding HLA-DR1 chains analogous to H and L chains of class I molecules. cDNA encoding the \(\alpha_1\) domain was ligated to the \(\beta\) chain cDNA, positioned between leader sequence and \(\beta_1\) domain cDNA, yielding a cDNA encoding an \(\alpha_1\beta_1\beta_2\) chain (see Fig. 2). Within the \(\alpha_1\beta_1\beta_2\) chain, amino acid 84 of the \(\alpha_1\) domain is attached directly to amino acid 1 of the \(\beta_1\) domain, without insertion of "spacer" residues. A transmembrane-associated \(\alpha_2\) construct was generated by splicing out the \(\alpha_1\) domain, and attaching leader sequence cDNA directly to the first amino acid.
of the α2 domain (amino acid 85 within the α chain). cDNA encoding a soluble α2 chain was generated by incorporation of a stop codon at the connecting peptide/transmembrane segment by site-directed mutagenesis (11). All cDNA constructs were ligated into the original pCD vectors, and the entire DNA insert was sequenced to confirm the desired product.

Abs. The following HLA class II-specific mAb were used in these studies: SG157 (obtained from Sanna Goyert, North Shore University Hospital, Manhasset, NY) (12); HU-26 (from Akemi Wakisaka, Hokkaido University School of Medicine, Sapporo, Japan) (13); LB3.1 (from Joan Gorga, Harvard University, Cambridge, MA) (14); and L243 (ATCC) (15).

Transfection. Cells of the DAP.3 subclone of class II-negative murine L cell fibroblasts were transfected using the calcium phosphate coprecipitation method as described (16). Transfections included 1 μg of the pSV2-neo plasmid and 20 μg each of plasmids containing DRAβ1-1, DRAα or DRα1-soluble chain cDNA. Control transfections, each including 1 μg pSV2-neo plasmid, included the following: 20 μg μg each of plasmids containing wild-type DRα and β chains; 40 μg of α1-β1-β2-containing plasmids; 40 μg of α2-containing plasmid. G418-resistant transfectant clones with stable surface expression of molecules recognized by SG157 were isolated by flow cytometric sorting as described (16).

T Cell Clones. HLA-DR1-restricted clone HA-1, specific for influenza A hemagglutinin H3 residues 307-319, was derived by in vitro stimulation as described (17), and kindly provided by A. Sette (Cytel Corp., San Diego, CA). Clone N3A9 (18), which recognizes residues 412-425 of the Mycobacterium leprae 65-kD heat shock protein, was kindly provided by T. H. M. Ottenhoff (University Hospital, Leiden, The Netherlands).

Peptides. Peptide HA, representing residues 307-319 of influenza A hemagglutinin H3, was purchased from Multiple Peptide Systems (San Diego, CA). Peptide HSP-65, containing residues 412-425 of the M. leprae 65-kD heat shock protein, was provided by Dr. D. C. Anderson (NeoRx Corp., Seattle, WA). Peptides were synthesized by solid phase methods and HPLC-purified before use.

T Cell Proliferation Assays. L cell transfectants expressing human MHC molecules were irradiated (3,000 rad) and seeded at 2 x 10⁵ cells/well in 96-well plates. Wells were pulsed overnight with peptide at the indicated concentrations, and washed twice with PBS before the addition of 5 x 10⁴ T cells/well. After 48 h culture, wells were pulsed for 14-16 h with 1.0 μCi [3H]Tdr, harvested onto glass filters, and assessed by liquid scintillation counting. Results are expressed as the mean counts per minute of triplicate wells. Individual replicates were within 15% of the mean.

Metabolic Cell Labeling. L cell transfectants were plated in 150 cm² tissue culture flasks at 10⁵ cells/flask and incubated overnight. Flasks were washed twice with sterile PBS before the addition of leucine-free Eagle's MEM containing 10% dialyzed FCS, 1 mM glutamine, penicillin, streptomycin, and 0.3 μCi/ml [3H]leucine (152 Ci/mmol, Amersham Corp., Arlington Heights, IL). Cells were incubated for 6 h at 37°C, rinsed twice with PBS, and detached using a rubber policeman into 5 ml PBS. Cells were centrifuged and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% NP-40, 0.02% NaN₃, 50 μg/ml PMSF, 50 μg/ml leupeptin, 50 μg/ml Pepstatin A) at 3 x 10⁷ cell equivalents/ml. Lysates were incubated at 4°C for 30 min, centrifuged at 14,000 rpm at 4°C for 30 min, and supernatants collected and stored at -20°C.

Immunoprecipitations and Gel Electrophoresis. 1 ml cell lysate was precleared by incubation with 300 μl Zetaphor G (Zymed Laboratories, Inc., South San Francisco, CA) and 5 μg MOPC 195 (Cappel Laboratories, West Chester, PA) overnight at 4°C. The Zetaphor G was pelleted and the supernatant was incubated for 6 h at 4°C with 25 μl protein G-Sepharose (Pharmacia, Piscataway, NJ) prearmed by incubation with mAb L243. The protein G-Sepharose was washed twice with TBS, twice with 0.5% Tween X-100 in TBS, then twice in TBS. After centrifugation, the protein G-Sepharose was resuspended in SDS-PAGE reducing sample buffer (0.0625 M Tris, 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, pH 6.8), boiled for 10 min, and vortexed before pelleting. Supernatants were analyzed on discontinuous SDS-polyacrylamide gels (5% stacking gel, 12.5% running gel). Gels were fixed for 30 min in 25% isopropanol/10% acetic acid, soaked for 30 minutes in Amplify (Amersham Corp.), dried, and exposed to film for 3-7 d at -70°C.

Results and Discussion

cDNAs encoding full-length DRα and DRβ chains were spliced to encode chains analogous to the H and L chains of class I MHC molecules (Fig. 1). cDNAs were designed

![Figure 1](https://example.com/figure1.png)

**Figure 1.** PCR splicing of cDNAs encoding DRα and β chains. AB and CD fragments were generated in independent PCR reactions, and the desired products were isolated from agarose gels. In a third PCR reaction, AB and CD fragments containing overlapping segments were annealed and extended to form AD fragments which incorporate unique restriction enzyme sites. (A) Formation of the α₁ construct. Splice site primer sequences: (a) GAGAGTCACCCCATATGATCCCACCCAT; and (b) CAGGAATCATGGGCTGTACCTCCAGAGGTAAC. (B) Formation of the α₁-β₁-β₂ construct. Splice site primer sequences: (a) TCCCCATTGCTGCGAGTATAGTT, and (c) ATCACCAATGGGCTGTACCTCCAGAGGTAAC.
to encode covalent attachment of the α1 domain COOH-terminus to the β1 domain NH2-terminus, based upon domain homologies with class I molecules (patterns of conserved sequence and disulfide bonds) (4). This 3+1-DR1 molecule, shown diagrammatically in Fig. 2, consists of a three domain α1-β1-β2 chain and single domain, membrane-bound α2 chain. The membrane distal, NH2-terminal domains forming the sites for interaction with peptide and TCR are thus contributed by the same polypeptide chain, analogous to class I. In addition, the 3+1-DR1 molecule no longer has the covalent association between α1 and α2 domains characteristic of class II molecules.

After cloning of cDNAs into the pCD expression vector, cotransfection of class II-negative murine L cells, and G418 selection, surface expression of molecules recognized by the human class II-specific mAb SG157 was detected. Analysis of uncloned transfectants receiving α2 and α1-β1-β2 chains demonstrated expression of the 3+1-DR1 molecule, whereas, transfection of vectors encoding either α2 or α1-β1-β2 chains alone did not result in cell surface expression of molecules detected by SG157 (data not shown). Also, there was no detectable cell surface expression of a 3+1-DR1 molecule using cDNAs encoding the α1-β1-β2 chain and a soluble α2 chain construct (data not shown). These results may reflect a requirement for interactions between transmembrane segments of α and β chains in formation of the class II dimer as recently described (19).

L cell clones with stable expression of the SG157 epitope were isolated by a single round of flow cytometric sorting and tested for recognition by additional mAbs recognizing HLA-DR1 (Fig. 3). mAbs L243 and LB3.1 each recognized 3+1-DR1 transfectant clones comparably to conventional DR1 transfectants that were selected for similar levels of SG157 expression. However, the epitope recognized by mAb HU26 was disrupted in the 3+1-DR1 transfectant relative to conventional DR1-expressing control transfectants. This HLA-DR epitope has previously been mapped to β chain position 4 (20), and thus may be disrupted in the 3+1-DR1 molecule by the covalent attachment of the α chain to the β chain NH2 terminus (Fig. 4). However, the overall conformation of the 3+1-DR1 construct appears to be comparable to conventional DR1, as demonstrated with mAbs SG157, LB3.1, and L243.

Although the 3+1-DR1 H and L chains fold into a structure that reconstitutes certain mAb recognition sites, the ability of this heterodimer to bind peptide and serve as a functional TCR ligand is a more meaningful test of conformation. Therefore, 3+1-DR1-expressing L cell clones were used as APC in proliferation assays with antigen-specific, DR1-restricted T cell clones. Clones HA-1 and N3A9, specific for peptides

Figure 2. Schematic representation of domain configurations in class I molecules, class II molecules, and the 3+1-DR1 construct.

Figure 3. mAb recognition of conventional DR1 and 3+1-DR1 molecules. Log fluorescence of 5,000 cells is shown for mAbs SG157, L243, LB3.1, and HU26.

Figure 4. Predicted structure of the class II binding region for peptide/TCR as proposed by Brown et al. (4). The covalent attachment between DRα1 and β1 domains in the 3+1-DR1 construct is shown. The location of amino acid residue 4 within the DR β1 domain is indicated.
derived from influenza hemagglutinin and *M. leprae* heat shock protein, proliferated comparably to peptide-pulsed transfectants expressing either conventional or 3+1-DR1 molecules (Fig. 5). Therefore, the class I-like domain configuration did not impair the ability of the 3+1-DR1 molecule to bind peptide in a conformation allowing T cell stimulation.

Class I molecules bind and present antigenic peptides that are shorter and more homogeneous in length compared to class II. Peptide truncation analog experiments as well as peptide elution studies with class I molecules indicate a strong preference for 8–10 residue peptides (21, 22). Within the class I molecule, pockets of conserved MHC residues at each end of the binding groove interact closely with COOH- and NH2-terminal peptide residues to confer this length preference. Our results demonstrate that 13 and 14 residue peptides can bind to, and be presented by, the 3+1-DR1 construct. Thus, the covalent linkage between α1 and β1 domains does not disrupt the ability to bind peptides of this length, and the “open-endedness” of the class II binding site does not appear to depend upon the lack of covalent attachment between NH2-terminal domains.

Metabolically labeled 3+1-DR1 molecules were immunoprecipitated and analyzed by SDS-PAGE. The results confirmed the expected molecular weights of the α2 (21 kD) and α1-β1-β2 (48 kD) chains (Fig. 6), and excludes the possibility that L cell clones expressing murine class II molecules were selected. The α2 chain, in contrast to β2-microglobulin, contains connecting peptide, transmembrane, and cytoplasmic segments, resulting in a correspondingly higher molecular weight.

Several pieces of evidence support the notion that the tertiary structures of class I and II molecules are highly homologous, including their overall domain composition, the presence of a single peptide binding site, patterns of conserved amino acids (4), the ability of certain T cells to recognize either class of MHC molecule (23), and the results of a detailed mutational analysis of the murine class II α chain (24). Indeed, preliminary reports of the HLA-DR1 structure suggest the overall validity of this model (7). However, differences in domain configuration could translate into important differences in quaternary protein structure, flexibility, or function. The present data supports the concept that class II assumes a conformation similar to class I, by demonstrating that HLA-DR1 conformation is not dramatically altered upon reorganization of its linear amino acid sequence into H and L chains analogous to class I.

The different domain configurations of class I and II MHC molecules may have evolved because they provide distinct functional properties related to antigen presentation. Certain MHC functions may be permitted, or quantitatively altered, by domain configuration, including peptide selection, kinetics of peptide binding and release, chaperone protein interactions, recycling of surface-expressed molecules, and “retrograde” signaling via the MHC molecule to the APC. The expression of MHC molecules with identical linear sequence but differing domain configurations allows direct testing of the functional correlates of domain organization, independent of linear amino acid sequence.

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**Figure 5.** Proliferation of DR1-restricted T cell clones in response to synthetic peptide presented by L cell transfectants. (A) Clone HA-1 response to peptide HA307-319; (B) Clone N3A9 response to peptide HSP65412-425. Incorporation of [3H]Tdr, in cpm, at the peptide concentrations indicated, was measured during the last 14 h of a 60-h incubation.

**Figure 6.** SDS-PAGE analysis of mAb L243 immunoprecipitates using transfectants expressing conventional DR1 and 3+1-DR1 molecules. (Lane 1) Neo transfectant (no class II expression); (lane 2) conventional DR1 transfectant; (lane 3) 3+1-DR1 transfectant. (MW standards) 14C-labeled control proteins.
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