Major histocompatibility complex (MHC) class II molecules are membrane-anchored heterodimers on the surface of antigen-presenting cells that bind the T cell receptor, initiating a cascade of interactions that results in antigen-specific activation of clonal populations of T cells. Susceptibility to multiple sclerosis is associated with certain MHC class II haplotypes, including human leukocyte antigen (HLA) DR2. Two DRB chains, DRB5*0101 and DRB1*1501, are co-expressed in the HLA-DR2 haplotype, resulting in the formation of two functional cell surface heterodimers, HLA-DR2a (DRA*0101, DRB5*0101) and HLA-DR2b (DRA*0101, DRB1*1501). Both isotypes can present an immunodominant peptide of myelin basic protein (MBP-(84–102)) to MBP-specific T cells from multiple sclerosis patients. We have previously demonstrated that the peptide binding/T cell recognition domain from rat MHC class II (α1 and β1 domains) could be expressed as a single exon for structural and functional characterization: Burrows, G. G., Chang, J. W., Bächinger, H.-P., Bourdette, D. N., Wegmann, K. W., Offner, H., and Vandenbark A. A. (1999) Protein Eng. 12, 771–778; Burrows, G. G., Adlard, K. L., Bebo, B. F., Jr., Chang, J. W., Tenditnyy, K., Vandenbark, A. A., and Offner, H. (2000) J. Immunol. 164, 6366–6371.

Single-chain human recombinant T cell receptor ligands (RTLs) of ~200 amino acid residues derived from HLA-DR2 were designed using the same principles and have been produced in Escherichia coli with and without amino-terminal extensions containing antigenic peptides. Structural characterization using circular dichroism predicted that these molecules retained the antiparallel β-sheet platform and antiparallel α-helices observed in the native HLA-DR2 heterodimer. The proteins exhibited a cooperative two-state thermal unfolding transition, and DR2-derived RTLs with a covalently linked MBP peptide (MBP-(85–99)) showed increased stability to thermal unfolding relative to the empty DR2-derived RTLs. These novel molecules represent a new class of small soluble ligands for modulating the behavior of T cells and provide a platform technology for developing potent and selective human diagnostic and therapeutic agents for treatment of autoimmune disease.

The pathogenesis of a variety of human diseases including multiple sclerosis, rheumatoid arthritis, diabetes, autoimmune uveitis, transplant rejection, chronic beryllium disease, and graft-versus-host disease appear to involve antigen-specific CD4+ T cells (1–5). It is thought that pathogenic T cells home to the target tissue where autoantigen is present and, after local activation, selectively produce Th1 lymphokines (6). This cascade of events leads to the recruitment and activation of lymphocytes and monocytes that ultimately destroy the target tissue (7).

Activation of CD4+ T cells in vivo is a multistep process initiated by co-ligation of the TCR and CD4 by the MHCII-class II-peptide complex present on APC (signal 1) as well as costimulation through additional T cell surface molecules such as CD28 (signal 2). Ligation of the TCR in the absence of costimulatory signals has been shown to disrupt normal T cell activation, inducing a range of responses from anergy to apoptosis (4–6). Within the context of this model of T cell activation, a direct approach toward Ag-driven immunosuppression would be to present the complete TCR ligand, Ag in the context of MHC, in the absence of costimulatory signals that are normally provided by specialized APCs. Toward the long term goal of targeted antigen-driven immunosuppression of pathogenic T cells, we have developed a family of novel recombinant TCR ligands (RTLs) that consist of the β1 and α1 domains of human MHC class II molecules. RTLs comprising the bound ligand/T cell recognition domain from rat MHC class II have been expressed as single exons (8), and these RTLs loaded with soluble peptide Ag had potent inhibitory activity on encephalitogenic T cells (9). The work on the rat system was recently extended when we described the in vivo and in vitro potency of single-chain constructs in which peptide Ag was genetically encoded at the 5’-end of the RTL exon, a design that favored specific loading and high occupancy of peptide Ag in the MHC binding cleft (10).

We developed our rat RTL constructs for clinical studies, treating experimental autoimmune encephalomyelitis, a paralytic, inflammatory, and sometimes demyelinating disease mediated by CD4+ T cells (1–5). It is thought that pathogenic T cells home to the target tissue where autoantigen is present and, after local activation, selectively produce Th1 lymphokines (6). This cascade of events leads to the recruitment and activation of lymphocytes and monocytes that ultimately destroy the target tissue (7).

To extend this work toward eventual human therapy, we have engineered molecules consisting of the α1 and β1 domains
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of the human MHC class II HLA-DR2 molecule genetically linked into a single polypeptide chain with and without covalently linked MBP-(85–99) peptide (Fig. 1).

We describe in this report the design, production, and purification of these HLA-DR2b-derived RTLs as well as biochemical and biophysical characterization demonstrating retention of properties required for TCR engagement. These findings anticipate the production of large amounts of these human recombinant TCR ligands for structural characterization and immunotherapeutic applications. Molecules with this design would be useful for studying binding specificity in vitro, for exploring primary TCR signaling events independent of co-stimulatory input, and for treating CD4+ T cell-mediated autoimmune disease in an epitope-specific manner.

EXPERIMENTAL PROCEDURES

Homology Modeling—Sequence alignment of MHC class II molecules from human, rat, and mouse species provided a starting point for our studies, as previously described (9). Graphic images were generated with the program Sybyl (Tripos Associates, St. Louis, MO) and an O2 work station (IRIX 6.5, Silicon Graphics, Mountain View, CA) using coordinates deposited in the Brookhaven Protein Data Bank (PDB) (Brookhaven National Laboratory, Upton, NY). Structure-based homology modeling was based on the refined crystallographic coordinates of human DR2 (19, 20) as well as DR1 (21, 22), murine I-E molecules (23), and scorpion toxins (24–26). Amino acid residues in human DR2 (PDB accession code 1BX2) (19) were used. Because a number of residues were missing and/or not located in the crystallographic data (19), the correct side chains were inserted, and the peptide backbone was modeled as a rigid body during structural refinement using local energy minimization.

Recombinant TCR Ligands (RTLs)—For production of the human RTLs, mRNA was isolated (Oligotex Direct mRNA mini kit; Qiagen, Inc., Valencia, CA) from L466.1 cells (gift from Dr. R. Karr, Searle Research and Development, St. Louis, MO) grown in RPMI media. First-strand cDNA synthesis was carried out using SuperScript II RNase H reverse transcriptase (Life Technologies, Inc.). Using the first-strand reaction as template source, the desired regions of the DRB*1501 and DRA*0101 DNA sequences were amplified by PCR using Taq DNA polymerase (Life Technologies, Inc.) with an annealing temperature of 55 °C. The primers used to generate β1 were 5' -attcctggtaggcccacgcatcagtttt-3' (huNcoI-1) and 5'-ggattgacatgttcttcttgatgctgctgctgctgctg-3' (huXhoI-1). The amplification reactions were gel-purified, and the desired bands were isolated (QIAquick gel extraction kit; Qiagen). The overhanging tails at the 5'-end of each primer added overlapping segments and restriction sites (NcoI and XhoI) at the ends of each PCR amplification product. The two chains were linked in a two-step PCR reaction. In the first step, 5 μl of each purified amplification product were added to a 50-μl primer-free PCR reaction and cycled 5 times at an annealing temperature of 55 °C. A 50-μl reaction mix containing the huNcoI-1 and huXhoI-1 primers was then added directly to the initial reaction and cycled 25 times at an annealing temperature of 50 °C. Taq DNA polymerase (Promega, Madison, WI) was used in each step. The final 100-μl reaction was gel-purified, and the desired huβ1a1 amplification product was isolated. The huβ1a1 insert was ligated with the PCR 2.1 plasmid vector (TA cloning kit, Invitrogen, Carlsbad, CA) and transformed into an INVA+ bacterial cloning host. PCR colony screening was used to select a single positive colony from which plasmid DNA was isolated (QIAprep Spin mini kit, Qiagen). Plasmid was cut with NcoI and XhoI restriction enzymes (New England BioLabs Inc., Beverly, MA) and gel-purified, and the huβ1a1 DNA fragment was isolated. The huβ1a1 DNA insert was ligated with NcoI/XhoI-digested pET-21d (+) plasmid expression vector (Novagen, Inc., Madison, WI) and transformed into BL21(DE3) expression host (Novagen). Bacterial colonies were selected based on PCR colony and protein expression screening. Plasmid DNA was isolated from positive colonies (QIAquick gel extraction kit, Qiagen) and sequenced with the T7 5'-taacgactcactataggctgg-3' and T7 terminator 5'-gtcgttagttgctcaggg-3' primers. After sequence verification, a single clone was selected for expression of the huβ1a1 peptide (RTL300).

A 30-amino acid huMBP-(85–99)-peptide linker cartridge was genetically inserted into the "empty" huβ1a1 (RTL300)-coding sequence between Arg-5 and Pro-6 of the β1 chain. The 90-base pair DNA sequence encoding the huMBP-(85–99) peptide and linker was inserted at position 1 of the RTL300 DNA construct in a three-step PCR reaction using Taq DNA polymerase (Promega). In the first step, pet-21d (+)/RTL300 plasmid was used as template in two separate PCR reactions. In the first reaction, the region from the start of the T7 priming site of the pet-21d (+) plasmid to the point of insertion within the huβ1a1 (RTL300) sequence was amplified with the primers 5'-gctacgacactataggctgg-3' (T7+) and 5'-aggctgccgaaggaagctccgtacccgacggtgctcaggg-3' (T7-). In the second reaction, the region from the point of insertion within the huβ1a1 (RTL300) sequence to the end of the T7-terminator-prime site was amplified with the primers 5'-tacgacactataggctgg-3' (T7+) and 5'-ggattgacatgttcttcttgatgctgctgctgctgctg-3' (huXhoI-1). In the second reaction, the region from the point of insertion within the huβ1a1 (RTL300) sequence to the end of the T7-terminator-prime site was amplified with the primers 5'-gctacgacactataggctgg-3' (T7+) and 5'-ggattgacatgttcttcttgatgctgctgctgctgctg-3' (T7-). In the second step, the amplified regions were primer extension amplified using the primers 5'-gtcgttagttgctcaggg-3' (T7+) and 5'-agtacgacactataggctgg-3' (T7-) and the PCR product was cloned into Lambda Zap Express (Stratagene, La Jolla, CA) phagemid. The insert was sequenced using the T7 5'-taacgacactcactataggctgg-3' and T7 terminator 5'-gtcgttagttgctcaggg-3' primers. After sequence verification, a single clone was selected for expression of the huβ1a1 peptide (RTL300).

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of each primer, creating the full-length huMBP-(85–99) peptide (RTL301) construct, which acted as template in the amplification step. The reaction was purified using agarose gel electrophoresis, and the desired huMBP-(85–99) peptide (RTL301) band was isolated. The PCR product was then cut with NcoI and XhoI restriction enzymes, gel-purified, ligated with a similarly cut pET-21d (+) plasmid expression vector, and transformed into a BL21(DE3) E. coli expression host. Transformants were screened for protein expression and the presence of the desired insert with a PCR colony screen. Plasmid DNA was isolated from several positive clones and sequenced. A single positive clone was selected for expression of the huMBP-(85–99) peptide (RTL301).

Repeated sequence analysis of pET-21d (+)/RTL300 and pET-21d (+)/RTL301 plasmid DNA constructs revealed the same thymine to cytosine single base pair deviation at position 358 and position 458 (RTL300 and RTL301). We used site-directed mutagenesis to revert the single base pair deviation at position 358 and position 458 (RTL300 and RTL301) to normal (RTL301 numbering). We used site-directed mutagenesis to revert the sequence to the GenBank accession number M60333, which resulted in an F150L mutation in the RTL301 and RTL302 molecules (RTL301 numbering). We used site-directed mutagenesis to revert the sequence to the GenBank accession number M60333. Two PCR reactions were performed using the pET-21d (+)/RTL300 and pET-21d (+)/RTL301 plasmids as template. For RTL300 the primers 5'-taa-tcagcgtgga-3' (T7 promoter) and 5'-taagcagtaacacacctgg-3' (huBA-F150L-3') were used. For RTL301 the primers 5'-ggaggtggtgccgctc-3' (huBA-F150L-5') and 5'-gcgtgtgctgccgg-3' (TTerminator) were used. The two resulting amplification products were gel-purified and isolated (QIAquick gel extraction kit, Qiagen), annealed, and amplified as described earlier, based on the complementary 5' and 3' ends of each of the PCR products. The final amplification reactions were gel-purified, and the desired PCR products were isolated. The NcoI and XhoI restriction sites flanking each were then used to subclone the RTL DNA constructs into fresh pET-21d (+) plasmid for transformation into BL21(DE3) competent cells and plasmid sequence verification. Positive clones were chosen for expression of the empty HLA-DR1-derived RTL302 molecule and the MBP-(85–99)-peptide-coupled RTL303 molecule (Fig. 2).

Expression and in Vitro Folding of the RTL Constructs—E. coli strain BL21(DE3) cells were transformed with the pET212d (+)/RTL vectors. Bacteria were grown in 1-liter cultures to mid-logarithmic phase (A600nm = 0.6–0.8) in Luria-Bertani (LB) broth containing carbenicillin (50 μg/ml) at 37 °C. Reombinant protein production was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside. After incubation for 3 h, the cultures were collected by centrifugation and stored at ~80 °C before processing. All subsequent manipulations of the cells were at 4 °C. The cell pellets were resuspended in ice-cold PBS, pH 7.4, and sonicated for 4 × 20 s with the cell suspension cooled in a salt/ice/water bath. The cell suspension was then centrifuged, the supernatant fraction was poured off, and the cell pellet was resuspended and washed three times in PBS and then resuspended in 20 mM ethanolamine, 6 M urea, pH 10, for 4 h. After centrifugation, the supernatant containing the solubilized recombinant protein of interest was collected and stored at 4 °C until purification. The recombinant proteins of interest were purified and concentrated by fast protein liquid chromatography ion-exchange chromatography using Source 30Q anion-exchange media (Amersham Pharmacia Biotech) in an XK26/20 column (Amersham Pharmacia Biotech) using a step gradient with 20 mM ethanolamine, 6 M urea, 1 M NaCl, pH 10. The proteins were dialyzed against 20 mM ethanolamine, pH 10, which removed the urea and allowed refolding of the recombinant protein. This step was critical. Basic buffers were required for all of the RTL molecular constructs to fold correctly, after which they could be dialyzed into PBS at 4 °C and concentrated by centrifugal ultrafiltration with Centricron 10 membranes (Amicon, Beverly, MA). For purification to homogeneity, a final step was included using size exclusion chromatography on Superdex 75 media (Amersham Pharmacia Biotech) in an HR16/50 column (Amersham Pharmacia Biotech). The final yield of purified protein varied between 15 and 30 mg/liter of bacterial culture.

Circular Dichroism and Thermal Transition Measurements—Circular dichroism (CD) spectra were recorded on a JASCO J-500A spectropolarimeter with an IF-500 digital interface and thermocellatically controlled quartz cells (Hellma, Mulheim, Germany) of 2-, 1-, 0.5-, 0.1- and 0.05-mm path length depending on peptide concentration. Data are presented as mean residue weight ellipticities. Calibration was regularly performed with (+)-10-camphorsulfonic acid (Sigma) to molar ellipticities of 7,780 and ~16,160 degree cm 2/dmol at 290.5 and 192.5 nm, respectively (27). In general, spectra were the average of 4–5 scans from 260 to 180 nm, recorded at a scanning rate of 5 nm/min with a 4-s time constant. Data were collected at 0.1-nm intervals. Spectra were averaged and smoothed using the built-in algorithms of the Jasco program, and buffer base lines were subtracted. Secondary structure was estimated with the program CONTIN (28). Thermal transition curves were recorded at a fixed wavelength of 222 nm. Temperature gradients from 5 to 90 or 95 °C were generated with a programmer-controlled circulating water bath (Lauda PM350 and RCS20D). Heating and cooling rates were between 12 and 18 °C/h. Temperature was monitored in the cell with a thermistor and digital thermometer (Omega Engineering), recorded and digitized on an XY plotter (HP7090A, Hewlett Packard), and stored on disc. The transition curves were normalized to the fraction of the peptide folded (F) using the standard equation $F = (U - U_n)/(U_u - U_n)$, where $U_u$ and $U_n$ represent the ellipticity values for the fully folded and fully unfolded species, respectively, and $U$ is the observed ellipticity at 222 nm.

RESULTS

Homology Modeling—Previous protein engineering studies describing RTLs derived from the α1 and β1 domains of rat MHC class II RT1.B (9) provided a starting point for designing
and refining human RTLs. Homology modeling studies of the heterodimeric MHC class II protein HLA-DR2 and, specifically, the α1 and β1 segments of the molecule that comprise the antigen binding domain were conducted based on the crystal structures of human DR (19–22). In our modeling studies we focused on three facets of the source protein organization and structure: 1) the interface between the membrane-proximal surface of the β-sheet platform and the membrane distal surfaces of the α2 and β2 Ig-fold domains, 2) the internal hydrogen bonding of the α1 and β1 domains, which comprise the peptide binding/TCR recognition domain, and 3), the surface of the RTLs, which was expected to interact with the TCR.

Side-chain densities for regions that correspond to a primary sequence between the β1 and β2 domains of human DR and murine I-EK showed evidence of disorder in the crystal structures (19–23), supporting the notion that these serve as linker regions between the two domains, with residue side chains having a high degree of freedom of movement in solution. High resolution crystals of MHC class II DR1 and DR2 (19–22) contained a large number of water molecules between the membrane proximal surface of the β-sheet platform and the membrane distal surfaces of the α2 and β2 Ig-fold domains. We quantified the surface area of interaction between domains by creating a molecular surface for the β1α1 and α2β2 Ig-fold domains with an algorithm developed by Michael Connolly (29) using the crystallographic coordinates for human DR2 available from the Brookhaven Protein Data Bank (1BX2). In this algorithm the molecular surfaces are represented by “critical points” describing holes and knobs. Holes (maxima of a shape function) are matched with knobs (minima). The surface areas of the α1β1 and α2β2-Ig-fold domains were calculated independently, defined by accessibility to a probe of radius 0.14 nm, about the size of a water molecule. The surface area of the MHC class II αβ-heterodimer was 160 nm², whereas that of the RTL construct was 90 nm² and that of the α2β2-Ig-fold domains was 90 nm². Approximately 15 nm² (19%) of the RTL surface was buried by the interface with the Ig-fold domains in the MHC class II αβ-heterodimer.

Human, rat, and murine MHC class II α chains share 30% identity, and the β chains share 35% identity. The backbone traces of the structures solved using x-ray crystallography showed strong homology when superimposed, implying an evolutionarily conserved structural motif (data not shown). The variability between the molecules is primarily within the residues that delineate the peptide binding groove, with sidechain substitutions designed to allow differential antigenic-peptide binding. The α1 and β1 domains of HLA-DR showed an extensive hydrogen-bonding network and a tightly packed and buried hydrophobic core. This tertiary structure appears similar to the molecular interactions that provide structural integrity and thermodynamic stability to the α-helix/β-sheet scaffold characteristic of scorpion toxins (24–26). The β1 domain of MHC class II molecules contains a disulfide bond that covalently couples the carboxyl-terminal end to the first strand of the anti-parallel β-sheet platform contributed by the β1-domain. This structure is conserved among MHC class II molecules from rat, human, and mouse and is conserved within the α2 domain of MHC class I. It appears to serve a critical function, acting as a “linchpin” that allows primary sequence diversity in the molecule while maintaining its tertiary structure. Additionally, a “network” of conserved aromatic side chains (30) appear to stabilize the RTLs (data not shown). Our studies predicted the antigen binding domain would remain stable in the absence of the α2 and β2 Ig-fold domains.

Expression and Production of RTLs—Novel genes were constructed by splicing the sequence encoding the amino terminus of HLA-DR2 α1 domain to the sequence encoding the carboxyl terminus of the β1 domain. We use the nomenclature RTL (recombinant TCR ligand) for proteins with this design. In this study we present experiments that used the empty RTL with the native sequence (RTL302), a covalent construct that contained the human MBP-(85–99) antigenic peptide (RTL303), and versions of these molecules (RTL300, empty; RTL301, containing MBP-(85–99)) that had a single phenylalanine to leucine alteration (F150L, RTL303 numbering) that eliminated biological activity (Fig. 2; See “Discussion”). Earlier work demonstrated that the greatest yield of material could be readily obtained from bacterial inclusion bodies, refolding the protein after solubilization and purification in buffers containing 6 M urea (9). Purification of the RTLs was straightforward and included ion exchange chromatography followed by size exclusion chromatography (Fig. 3). After purification, the protein was dialyzed against 20 mM ethanolamine, pH 10.0, which removed the urea and allowed refolding of the recombinant protein. This step was critical. Basic buffers were required for all of the RTL molecular constructs to fold correctly, after which they could be dialyzed into PBS at 4 °C for in vivo studies. The final yields of empty and antigenic peptide-coupled RTLs was ~15–30 mg/liter culture.

Biochemical Characterization—Oxidation of cysteines 46 and 110 (RTL303 amino acid numbering, corresponding to DR2 β chain residues 15 and 79) to reconstitute the native disulfide bond was demonstrated by a gel shift assay (Fig. 4) in which identical samples with or without the reducing agent β-mercaptoethanol were boiled 5 min before SDS-polyacrylamide gel
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electrophoresis. In the absence of β-mercaptoethanol, disulfide bonds are retained, and proteins typically demonstrate a higher mobility during electrophoresis through acrylamide gels due to their more compact structure. Representative examples of this analysis are shown for the empty RTL300 and RTL302 and the MBP-coupled RTL301 and RTL303 molecules (Fig. 4). All of the RTL molecules produced showed this pattern, indicating the presence of the native conserved disulfide bond. These data represent a primary confirmation of the conformational integrity of the molecules.

**Structural Analysis**—CD demonstrated the highly ordered secondary structures of RTL 302 and RTL303 (Fig. 5; Table I). RTL303 contained ~38% α-helix, 33% β-strand, and 29% random coil structures. Comparison with the secondary structures of class II molecules determined by x-ray crystallography (19–23) provided strong evidence that RTL303 shared the β-sheet platform, anti-parallel α-helix secondary structure common to all class II antigen binding domains (Table I).

Structure loss upon thermal denaturation indicated that the RTLs used in this study are cooperatively folded (Fig. 6). The temperature ($T_m$) at which half of the structure is lost for RTL303 is ∼78 °C, which is similar to that determined for the rat RTL.B MHC class II-derived RTL201 (9). RTL302, which does not contain the covalently coupled Ag-peptide, showed a 32% decrease in α-helical content compared with RTL303 (Table I). This decrease in helix content was accompanied by a decrease in thermal stability of 36% (28 °C) compared with RTL303, demonstrating the stabilization of the RTL molecule and, by inference, the antigen presentation platform of MHC class II molecules that accompanies peptide binding. Again, this trend is similar to what has been observed using rat RTL molecules (9), although the stabilization contributed by the covalently coupled peptide is ∼3-fold greater for the human RTLs. The F150L-modified RTL301 molecule showed a 48% decrease in α-helical content (Table I) and a 21% (16 °C) decrease in thermal stability (Fig. 6) compared with RTL303. RTL300, which had the F150L modification and lacked the covalently coupled Ag-peptide, showed cooperativity during structure loss in thermal denaturation studies but was extremely unstable ($T_m = 48 ^\circ C$) relative to RTL302 and RTL303, and the secondary structure could not be determined from the CD data (Figs. 5 and 6; Table I).

An explanation for the thermal stability data comes from molecular modeling studies using the coordinates from DR2a and DR2b MHC class II crystal structures (PDB accession codes 1FV1 and 1BX2; Refs. 19 and 20). These studies demon-

![Fig. 4. Purified and refolded DR2-derived RTLs have a native disulfide bond. Samples of RTLs were boiled for 5 min in Laemmli sample buffer with or without the reducing agent β-mercaptoethanol (β-ME) and then analyzed by SDS-polyacrylamide gel electrophoresis (12%). Non-reduced RTLs (~ lanes) have a smaller apparent molecular weight than reduced RTLs (+ lanes), indicating the presence of a disulfide bond. The first and last lanes show the molecular weight standards of carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa). RTLs (+/β-mercaptoethanol) are as indicated.](image)

![Fig. 5. Circular dichroism shows the DR2-derived RTLs have highly ordered structures. CD measurements were performed at 20 °C on a Jasco J-500 instrument using 0.1-mm cells from 260 to 180 nm. Concentration values for each protein solution were determined by amino acid analysis. Buffer composition was 50 mM potassium phosphate, 50 mM sodium fluoride, pH 7.8. Analysis of the secondary structure was performed using the variable selection method (37).](image)

![Fig. 6. Thermal denaturation shows a high degree of cooperativity and stability of the DR2-derived RTLs. CD spectra were monitored at 222 nm as a function of temperature. The heating rate was 10 °C/h. The graph charts the percent of unfolding as a function of temperature. 1.0 corresponds to the completely unfolded structure.](image)

**Table I**

| Molecule | Description | α-Helix | β-Sheet | Other | Total | Reference |
|----------|-------------|---------|---------|-------|-------|-----------|
| RTL201   | RTL.B β1a1 Gp-MBP72–89 | 0.28    | 0.39    | 0.33  | 1.0   | 9         |
| RTL300   | DR2 β1a1 (F150L)  | —       | —       | —     | ND    | This paper|
| RTL301   | DR2 β1a1/mb-MBP85–99 (F150L) | 0.20    | 0.35    | 0.46  | 1.0   | This paper|
| RTL302   | DR2 β1a1 (empty) | 0.28    | 0.31    | 0.43  | 1.0   | This paper|
| RTL303   | DR2 β1a1/mb-MBP85–99 | 0.38    | 0.33    | 0.29  | 1.0   | This paper|
| 1BX2     | DR2 (DRA*0101, DRB1*1501) | 0.32    | 0.37    | 0.31  | 1.0   | 19        |
| 1AQD     | DR1 (DRA*0101, DRB1*0101) | 0.32    | 0.37    | 0.31  | 1.0   | 22        |
| 11AK     | Murine I-Aa | 0.34    | 0.37    | 0.29  | 1.0   | 23        |
| 11EA     | Murine I-Ea | 0.27    | 0.31    | 0.42  | 1.0   | 23        |

*β-Sheet includes parallel and anti-parallel β-sheet and β-turn structures.

F150L is based on RTL303 numbering (See Fig. 2).

RTL300 CD data could not be fitted using the variable selection method.
strated that Phe-150 is a central residue within the hydrophobic core of the RTL structure (Fig. 7; Table II), part of a conserved network of aromatic side chains that appears to stabilize the secondary structure motif that is completely conserved in human class II molecules and is highly conserved between rat, mouse, and human MHC class II. The motif couples three anti-parallel β-sheet strands to a central unstructured stretch of polypeptide between two α-helical segments of the α1 domain. The structural motif is located within the α1 domain and “caps” the α1 domain side at the end of the peptide binding groove where the amino terminus of the bound antigenic peptide emerges.

We have constructed soluble single-chain RTL molecules derived from the antigen-binding β1 and α1 domains of human MHC class II molecule DR2. The RTLs lack the α2 domain, the β2 domain known to bind to CD4, and the transmembrane and intra-cytoplasmic sequences. The reduced size of the RTLs gave us the ability to express and purify the molecules from bacterial inclusion bodies in high yield (15–30 mg/liter of cell culture). The RTLs refolded upon dialysis into PBS and had excellent solubility in aqueous buffers.

The data presented demonstrate clearly that the human DR2-derived RTL302 and RTL303 retain structural and conformational integrity, consistent with crystallographic data regarding the native MHC class II structure. MHC class II molecules form a stable heterodimer that binds and presents antigenic peptides to the appropriate T cell receptor (Fig. 1). Although there is substantial structural and theoretical evidence to support this model (21–23, 31, 32), the precise role that contextual information provided by the MHC class II molecule plays in antigen presentation, T cell recognition, and T cell activation remains to be elucidated. Our approach used rational protein engineering to combine structural information from x-ray crystallographic data with recombinant DNA technology to design and produce single-chain TCR ligands based on the natural MHC class II peptide binding/T cell recognition domain. In the native molecule this domain is derived from portions of the α and β polypeptide chains that fold together to form a tertiary structure, most simply described as a β-sheet platform upon which two anti-parallel helical segments interact to form an antigen binding groove. A similar structure is formed by a single exon encoding the α1 and α2 domains of MHC class I molecules, with the exception that the peptide binding groove of MHC class II is open-ended, allowing the engineering of single-exon constructs that incorporate the peptide binding/T cell recognition domain and an antigenic peptide ligand (33).

From a drug-engineering and design perspective, this prototypic molecule represents a major breakthrough. Development of the human RTL molecules described in this report separates for the first time the peptide binding αβ1 domains from the platform αβ2 Ig-fold domains, allowing studies of their biochemical and biological properties independently, both from each other and from the vast network of information exchange that occurs at the cell surface interface between APC and T cell during MHC/peptide engagement with the T cell receptor. Although a pharmacological approach to T cell-mediated diseases is still in its infancy, development of human RTL molecules described in this report will allow careful evaluation of the specific role played by a natural TCR ligand independent from the platform αβ2 Ig-fold domains of MHC class II.

When incubated with peptide-specific Th1 cell clones in the absence of APC or costimulatory molecules, RTL303 initiated a subset of quantifiable signal transduction processes through the TCR. These included rapid ζ chain phosphorylation, calcium mobilization, and reduced extracellular signal-regulated kinase activity as well as interleukin-10 production. The addition of RTL303 alone did not induce proliferation. T cell clones pretreated with cognate RTLs before restimulation with APC and peptide had a diminished capacity to proliferate and se-
crete interleukin-2 and secreted less IFN-γ. Importantly, interleukin-10 production persisted. These data elucidate for the first time the early signaling events induced by direct engagement of the external TCR interface in the absence of signals supplied by co-activating molecules. The detailed biological and immunological implications of RTL treatment on T cell behavior are described in a recently submitted manuscript.3

Modeling studies have highlighted a number of interesting features regarding the interface between the α1α1 and α2β2-Ig-fold domains. The α1 and β1 domains showed an extensive hydrogen-bonding network and a tightly packed and buried hydrophobic core. The RTL molecules, composed of the α1 and β1 domains, may have the ability to move as a single entity independent from the α2β2-Ig-fold “platform.” Flexibility at this interface may be required for freedom of movement within the α1 and β1 domains for binding/exchange of peptide antigen. Alternatively or in combination, this interaction surface may play a potential role in communicating information about the MHC class II/peptide molecules interaction with TCRs back to the APC.

Critical analysis of the primary sequence of amino acid residues within two helical turns (7.2 residues) of the conserved cysteine 110 (RTL303 numbering) as well as analysis of the β-sheet platform around the conserved cysteine 46 (RTL303 numbering) reveal a number of interesting features of the molecule, the most significant being very high diversity along the peptide binding groove face of the helix and β-sheet platform. Interestingly, the surface-exposed face of the helix composed of residues Leu-99, Glu-100, Arg-103, Ala-104, Asp-107, Arg-111, and Tyr-114 (Fig. 1) is conserved in all rat, human, and mouse class II and may serve an as yet undefined function. Site-directed mutagenesis studies using the novel RTL molecules described in this report will allow careful dissection of the specific role played by each of these residues.

Cooperative processes are extremely common in biochemical systems. The reversible transformation between an α-helix and a random coil conformation is easily quantified by circular dichroism. Once a helix is started, additional turns form rapidly until the helix is complete. Likewise, once it begins to unfold it tends to unfold completely. A normalized plot of absorption of circularly polarized light at 222 nm versus temperature (melting curve) was used to define a critical Tm for each RTL molecule. The melting temperature was defined as the midpoint of the decrease in structure loss calculated from the loss of absorption of polarized light at 222 nm.

Our data support a hypothesis that peptide binding keeps the α1 and β1 domain helices of MHC class II molecules in “relative register,” completing and stabilizing a structure for binding to a subset of the T cells positively selected in the thymus. Because T cells undergo positive selection in the thymus before maturation to the point where they are allowed to the periphery, it is straightforward to propose that empty RTL molecules wouldn’t bind to the TCR on these cells.

Because of their size and biochemical stability, RTLs will serve as a platform technology for development of protein drugs with engineered specificity for particular target cells and tissues. Our primary focus in engineering these molecules was for controlling T cell behavior and phenotype. Ligands that bind T cell receptors in an antigen-specific manner would have a number of practical applications including the ability to target, label, and/or purify antigen-specific T cells, to activate T cells in combination with other molecules, and to treat conditions mediated by antigen-specific T cells.

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