Published in final edited form as:

J Chem Technol Biotechnol. 2005 January ; 80(1): 2–12. doi:10.1002/jctb.1086.

Rationally designed mutations convert complexes of human recombinant T cell receptor ligands into monomers that retain biological activity

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Abstract

Single-chain human recombinant T cell receptor ligands derived from the peptide binding/TCR recognition domain of human HLA-DR2b (DRA*0101/DRB1*1501) produced in *Escherichia coli* with and without amino-terminal extensions containing antigenic peptides have been described previously. While molecules with the native sequence retained biological activity, they formed higher order aggregates in solution. In this study, we used site-directed mutagenesis to modify the β -sheet platform of the DR2-derived RTLs, obtaining two variants that were monomeric in solution by replacing hydrophobic residues with polar (serine) or charged (aspartic acid) residues. Size exclusion chromatography and dynamic light scattering demonstrated that the modified RTLs were monomeric in solution, and structural characterization using circular dichroism demonstrated the highly ordered secondary structure of the RTLs. Peptide binding to the 'empty' RTLs was quantified using biotinylated peptides, and functional studies showed that the modified RTLs containing covalently tethered peptides were able to inhibit antigen-specific T cell proliferation *in vitro*, as well as suppress experimental autoimmune encephalomyelitis *in vivo*. These studies demonstrated that RTLs encoding the Ag-binding/TCR recognition domain of MHC class II molecules are innately very robust structures, capable of retaining potent biological activity separate from the Ig-fold domains of the progenitor class II structure, with prevention of aggregation accomplished by modification of an exposed surface that was buried in the progenitor structure.

Keywords

major histocompatibility complex; protein structure; drug design; autoimmunity

INTRODUCTION

Antigen-specific CD4⁺ T cells appear to be a central component in the pathogenesis of a variety of human diseases including multiple sclerosis (MS), rheumatoid arthritis (RA), diabetes, sarcoidosis, autoimmune uveitis, chronic beryllium disease, transplant rejection and graft-versus-host disease (GVHD).^{1–5} Antigen-specific CD4⁺ T cells that mediate autoimmune disease home to the target tissue where autoantigen is present and selectively produce T-helper type 1 lymphokines.⁶ This cascade of events leads to the recruitment and activation of lymphocytes and monocytes that ultimately destroy the target tissue.⁷ Antigen-driven activation of CD4⁺ T cells is a multi-step process initiated by co-ligation of the TCR and CD4 by the MHC class II/peptide complex present on APC (signal 1), as well as co-stimulation through additional T cell surface molecules such as CD28 (signal 2). The classic experiment by Quill and Schwartz⁸ demonstrated that stimulation through the TCR by MHC class II/peptide in the absence of co-stimulation, rather than being a neutral event, induced a state of unresponsiveness to subsequent optimal antigen presentation, a phenomenon termed anergy.^{8,9} Subsequently, 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.^{9–12}

MHC class II molecules are membrane-bound glycoproteins made up of non-covalently associated α - and β -polypeptide subunits. Each subunit consists of a short cytoplasmic tail, a single membrane-spanning sequence, and two extracellular domains. X-ray crystallographic studies have demonstrated that peptides from processed antigen bind to MHC molecules in the membrane distal pocket formed by the β 1 and α 1 domains.^{13,14} Moreover, the β 2 domain contains a CD4 binding region that co-ligates CD4 when the α 1 and β 1 domains with associated antigenic peptide interact with the TCR $\alpha\beta$ heterodimer, whereas the α 2 domain appears to contribute to ordered oligomerization in T cell activation.¹⁵ Complexes of MHC/antigen have been purified as detergent extracts of lymphocyte membranes,¹⁶ and as associated recombinant proteins using baculovirus and bacterial expression systems.^{17–21} These two-chain, four-domain molecular complexes, after loading with selected peptide epitopes, have been demonstrated to interact with T cells in an antigen-specific manner.^{13,14,19,22–24} In some cases, in the absence of co-stimulation, these complexes induced antigen-specific apoptosis rather than anergy.²⁰ Desbarats *et al* showed that cross-linking CD4 can enhance Fas (CD95) expression and activate apoptotic cell death.²⁵ Taken together, these findings would indicate that the difference in downstream information processing in the activated T cells is due to different signals derived from the context in which the MHC/antigen complex interacted with the TCR- alone, versus with co-ligation of the TCR and CD4.

To develop a simple and effective agent that could bind selectively to the TCR, we have developed a family of novel recombinant TCR ligands (RTLs) that consist of the α 1 and β 1 domains of MHC class II molecules, the antigen binding/TCR recognition domain, genetically linked into a single polypeptide chain.^{26,27} Molecules with this design have been demonstrated to be useful for studying binding specificity *in vitro*,²⁸ for exploring primary TCR signaling events independent of co-stimulatory input associated with the MHC class II α 2 and β 2 domains or with other molecules expressed by antigen presenting cells,¹² and for treating CD4⁺ T cell-mediated autoimmune disease in an MHC II/epitope-specific manner.^{11,29}

In recently described protein engineering studies of RTLs derived from HLA-DR2 (DRB1*1501/DRA*0101),²⁷ we found that DR2-derived molecules formed aggregates, with approximately 10% of the molecules in the form of stable dimers and the remainder purified in the form of higher-order structures above 300 000 daltons.²⁷ While these aggregates

retained biological activity,^{11,29} we sought to redesign surface features of the RTLs with the goal of converting these multimeric complexes into monomers with retention of biological activity, as a prerequisite toward eventual use as a human therapeutic in treatment of multiple sclerosis. Here, we report that the modified monodisperse molecules retained the ability to bind Ag-peptides, inhibit T cell proliferation in an Ag-specific manner and were able to treat experimental autoimmune encephalomyelitis (EAE) *in vivo*.

EXPERIMENTAL PROCEDURES

Homology modeling

Much of the logic for dissecting the molecules has been previously described.^{26,27} Sequence alignment of MHC class II molecules from human, rat and mouse species provided a starting point for our studies and graphic images were generated with the program Sybyl 6.9 (Tripos Associates, St Louis, MO) on an O2 workstation (IRIX 6.5, Silicon Graphics, Mountain View, CA) using coordinates deposited in the Brookhaven Protein Data Bank (Brookhaven National Laboratories, Upton, NY). Structure-based homology modeling was based on the refined crystallographic coordinates of human HLA-DR2,^{30,31} as well as DR1,^{32,33} murine I-E^k molecules,³⁴ and scorpion toxins.^{35–37} Amino acid residues in human HLA-DR2 (PDB accession code 1BX2) were used. This structure was determined by single wavelength diffraction and molecular replacement (AmoRe XRay/NMR structure refinement package, CNRS, France) using HLA-DR1 as a starting structure (PDB accession code 1DLH).³⁸ The following residues were either missing or had missing atoms in the final structure: chain A; K2, M36, K38, K39, E46, N78, R100, E101; chain B: E22, E35, E52, E59, K65, E69, P108, R189 (1BX2 numbering).³⁰ For these residues the correct side chains were inserted and the peptide backbone was modeled as a rigid body during structural refinement using local energy minimization.

RTL structural modification

De novo synthesis of human HLA-DR2 derived RTLs has been previously described.²⁷ Site-directed mutagenesis was used to replace hydrophobic residues on the solvent-accessible surface of the β -sheet platform of the RTLs with polar (serine) or charged (aspartic acid) residues. The modification was performed by using the QuickChangeTM site-directed mutagenesis method as described by Stratagene (La Jolla, CA). In brief, PCR reaction with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was performed by using RTL302 or RTL303 as template and two synthetic oligonucleotide primers containing the desired mutation(s). For example, a pair of mutation primers for RTL320 were (1) forward primer: 5'-

GGCGAGTCATCAAAGAAGAACATAGCATCAGCCAGAGCGAGAGTTATAGTAAT
CCTGACCAATC-3'; (2) backward primer: 5'-GATTGGTCA-

GGATTACTATAACTCTCGCTCTGGCTGATGCTATGTTCTTCTTTGATGACTC-3';

and a pair of mutation primers for RTL340 were (1) forward primer: 5'-

GGCGAGTCATCAAAGAAGAACAT-

GACATCGACCAGGACGAGGACTATGACAATCCTGACCAATC-3'; (2) backward

primer: 5'-GATTGGTCAGGATTGTCATAGTCCTCGTC-

CTGGTCGATGTCATGTTCTTCTTTGATGACTC-3'. The oligonucleotide primers, each

complementary to the opposite strand of template, were extended during 19 temperature cycles by means of *Pfu* DNA polymerase at an annealing temperature of 55 °C. Upon incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the PCR product was treated with *DpnI* endonuclease to digest the parental DNA template and to select for mutants containing the DNA sequence of interest. The nicked plasmid DNA incorporating the desired mutation(s) was then transformed into *Escherichia Coli* BL21(DE3) as an expression host (Novagen,

Madison, WI). Colonies were screened and cells containing plasmid with the desired mutation(s) were used for plasmid purification using QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA). The purified plasmid DNA was then digested with *Nco*I and *Xho*I to confirm the efficiency of mutation. Finally, the desired plasmids were sequenced with the (T7) 5'-TAATACGACTCACTATAGGG-3' and (T7 terminal) 5'-GCTAGTTATTGCTCAGCGG-3' primers to confirm mutations of interest.

Expression and refolding of soluble RTL molecules

Expression, purification and refolding of human HLA-DR2 derived RTLs was previously described.²⁷ A number of modifications have been made in the protocol to streamline production while maintaining or slightly increasing the yield of protein. Bacteria were grown in 1 dm³ cultures to mid-logarithmic phase (OD₆₀₀ = 0.6–0.7) in Luria–Bertani (LB) broth containing carbenicillin (50 µg cm⁻³) at 37 °C. Recombinant protein production was induced by addition of 0.5 mmol dm⁻³ isopropyl β-D-thiogalactoside (IPTG). After incubation for 4 h, the cells were harvested by centrifugation and stored at 4 °C (short-term) or –80 °C (long-term) before processing. All subsequent manipulations of protein purification were at 4 °C. The cell pellets were resuspended in lysis buffer (50 mmol dm⁻³ Tris-Cl, 0.1 mol dm⁻³ NaCl, 5 mmol dm⁻³ EDTA, pH 7.4). Lysozyme (10 mg cm⁻³ solution in lysis buffer; 1 mg per gram of cell pellet) was added, and the solution was incubated at room temperature for 30 min, swirling gently every 10 min. The cell suspension was then sonicated for 6 × 5 s with the cell suspension cooled in a salt ice water bath. The cell suspension was centrifuged (20 000 *g* for 10 min at 4 °C, Beckman J2-21, JA-14 rotor), the supernatant fraction was poured off, the cell pellet resuspended and washed two times in 100 cm³ lysis buffer containing 1% Triton X-100 and then one wash in lysis buffer without Triton X-100, and then resuspended in 100 cm³ Buffer A (20 mmol dm⁻³ ethanolamine, 6 mol dm⁻³ urea, pH 10), and stirred gently at 4 °C overnight. After centrifugation (40 000 *g* for 45 min at 4 °C, Beckman J2-21, JA-20 rotor), the supernatant containing the solubilized recombinant protein of interest was filtered (0.22 µm parasize, Stericup, Millipore) and stored at 4 °C until purification. The recombinant proteins of interest were purified and concentrated by FPLC ion-exchange chromatography using Source 30Q anion-exchange media (Pharmacia Biotech, Piscataway, NJ) in an XK26/20 column (Pharmacia Biotech), using a step gradient with buffer A and buffer B (20 mmol dm⁻³ ethanolamine/HCl, 6 mol dm⁻³ urea, 2 mol dm⁻³ NaCl, pH 10.0). Fractions containing the recombinant protein of interest were pooled and concentrated for size exclusion chromatography (SEC buffer, 20 mmol dm⁻³ ethanolamine, 6 mol dm⁻³ urea, 0.2 mol dm⁻³ NaCl, pH 10.0; column, Superdex 75, HR16/60). Fractions containing protein of interest were pooled and diluted with SEC buffer to OD₂₈₀ of 0.1. Proteins were dialyzed against 20 mmol dm⁻³ Tris-Cl at pH 8.5, which removed the urea and allowed refolding of the recombinant protein. Following dialysis, the proteins were concentrated by centrifugal ultrafiltration with Centricon 10 membranes (Amicon, Beverly, MA). For purification to homogeneity, a finish step was included using size exclusion chromatography (Superdex 75, HR16/60). The final yield of purified protein varied between 15 and 30 mg dm⁻³ of bacterial culture.

SDS-gel shift assay

Aliquots of purified protein sample were denatured by boiling for 5 min in Laemmli buffer with or without the reducing agent β-mercaptoethanol, and then analyzed by electrophoresis (12% SDS–PAGE). After electrophoresis, gels were stained with Coomassie Brilliant Blue (Sigma, St Louis, MO) and destained for observation of molecular weight shifting.

Dynamic light scattering

Dynamic light scattering (DLS) experiments were conducted with a DynaPro™ instrument (Protein Solutions, Inc, Charlottesville, VA). The protein samples in 20 mmol dm⁻³ Tris-Cl

buffer at pH 8.5 were filtered through 100 nm Anodisc membrane filter (Whatman, Clifton, NJ) at a concentration of 1.0 mg dm^{-3} and 20 mm^3 of filtered sample were loaded into a quartz cuvette and analyzed at 488 nm. Fifty spectra were collected at 4°C to get estimation of the diffusion coefficient and relative polydispersity of proteins in aqueous solution. Data were then analyzed by Dynamics software version 5.25.44 (Protein Solutions, Charlottesville, VA) and buffer baselines were subtracted. Data were expressed as the mean of the calculated hydrodynamic radius. Molecular weights of RTLs were calculated assuming a globular hydrated shape for the molecules using Dynamics software version 5.25.44 (Protein Solutions, Charlottesville, VA).

Circular dichroism (CD) and thermal denaturation analysis

CD analysis and thermal denaturation studies were performed as previously described.²⁷ In brief, recombinant proteins in 20 mmol dm^{-3} Tris-Cl buffer pH 8.5 were analyzed using an Aviv Model 215 CD spectrometer (Aviv Associates, Lakewood, NJ). Spectra were the average of four or five scans from 260 to 180 nm, recorded at a scanning rate of 5 nm min^{-1} with 4-s time constant. Data were collected at 0.5 nm intervals. Spectra were averaged and smoothed using built-in algorithms and buffer baselines were subtracted. Secondary structure was estimated using a deconvolution software package (CDNN version 2.1, Aviv Associates, Lakewood, NJ) based on the variable selection method.³⁹ CD versus temperature (thermal denaturation curve) was recorded at a fixed wavelength of 208 nm. Temperature gradients from 60 to 95°C were generated with a software controlled thermoelectric device to generate rising or falling temperature steps. Heating and cooling rates were between 10 and 12°C h^{-1} . The transition curves were normalized to 0 mdeg at 60°C and are plotted as the change in absorbance (mdeg) as a function of temperature.

Enzyme linked immunosorbant assay (ELISA)

Biotinylated MOG-35-55 peptide (Biot-MEVGWYRSPFSRVVHLYRNGK-OH), non-biotinylated MOG-35-55 and MBP-85-99 peptide (ENPVVHFFKNIVTPR-OH) were purchased from New England Peptide, Inc. (Fitchburg, MA). The purity of the peptides was verified by a reverse phase HPLC and mass identification was performed using MALDI-TOF to verify mass was within 0.1% of molecular weight expected. The peptides were lyophilized and stored at -80°C until use. Direct binding assay experiments were carried out in order to determine the ability of the RTLs to bind peptide and to determine the concentration of the biotinylated peptide at which all specific binding sites were saturated under the conditions used in our studies. ELISA plates (Maxisorp, Nunc, Rochester, NY) were coated with 50 mm^3 of protein at a concentration of $1 \mu\text{g cm}^{-3}$ in 20 mmol dm^{-3} Tris, pH 8.5 (50 ng of protein; ie 40 nmol dm^{-3}) overnight at 4°C , washed four times with wash solution (0.05% v/v: Tween 20, PBS, pH 7.4), and blocked with a Casein solution (BioFX, Owing Mills, MD) for 1.5 h at room temperature. Plates were then washed 4 $\times$ and 50 mm^3 of biotinylated peptide (serial dilutions) were added to the wells, RT, 1.5 h, and then washed 4 $\times$. Fifty mm^3 of a streptavidin-horseradish peroxidase conjugate (STR-HRP, 1:5000, DAKO, Glostrup, Denmark) in PBS was added to the wells and incubated at RT for 1.5 h then washed 4 $\times$ to remove unbound conjugate. Fifty mm^3 of HRP substrate (BioFX) was added for 45 min, RT. Reactions were stopped with Stop Solution (BioFX) and bound peptide was determined indirectly by reading the absorbance at 405 nm in an ELISA plate reader (Applied Biosystems, Molecular Devices, Sunnyvale, CA). A standard curve of STR-HRP concentration vs $\text{OD}_{405 \text{ nm}}$ was used to determine the concentration of bound peptide. To control nonspecific binding, wells were coated with 3% (w/v) non-fat dry milk (NFDM) in PBS and treated in the same way as the RTL-coated wells. In order to determine the time required to reach steady-state binding of the peptides to the proteins, ELISA plates were coated, washed and blocked as above and then biotinylated peptide in PBS/ 1 mmol dm^{-3} EDTA at pH 7.4 at $0.15 \text{ mmol dm}^{-3}$ was added at different times (0 to 36 h).

T cell clones and T cell proliferation assay

Antigen-specific T cell clones were selected from PBMC of an MS patient homozygous for HLA-DRB1*1501 as previously described.¹¹ In brief, selected antigen-specific T cell clones were subcloned by a limiting dilution method and subsequently evaluated for antigen-specific proliferation. The clone with the highest stimulation index (SI) was selected and continuously cultured in RPMI medium supplemented with 1% (v/v) human serum and 5 ng cm⁻³ IL-2. Clonality of cells was determined by RT-PCR, with a clone defined as a T cell population utilizing a single TCR V β gene. T cell clones were expanded by stimulation with 1 μ g cm⁻³ MOG-35-55 or MBP-85-99 peptide and 2 $\times$ 10⁵ irradiated (2500 rad) autologous PBMCs per well in a 96-well plate. The expanded T cells were maintained in 1% human serum RPMI containing 5 ng cm⁻³ IL-2. Fresh IL-2 was added twice a week and T cell clones were restimulated with irradiated (2500 rad) autologous PBMCs every 3 weeks. Antigen-specific T cell proliferations were performed periodically to verify the quality of the cells. For these assays, antigen-specific T cell clones were washed twice with RPMI medium and 5 $\times$ 10⁴ cells were re-seeded into each well in a 96-well plate and incubated in triplicate with 2 $\times$ 10⁵ freshly isolated and irradiated (2500 rad) autologous PBMCs with 10 μ g cm⁻³ of the desired peptide. Cells were incubated for 72 h with [³H]-thymidine added for the last 18 h. Cells were collected (Harvester 96; Tomtec, Hamden, CT) and radioactivity incorporated was quantified (1205 BS liquid scintillation counter; Wallac, Turku, Finland). Stimulation index (SI) was calculated by dividing the mean cpm of peptide-added wells by the mean cpm of the medium alone, control wells. For RTL treatment experiments, 8 μ mol dm⁻³ of the desired RTL was pre-incubated with the T cell clones for 72 h, following by two washes with RPMI media before the T cell proliferation assay was performed.

Mice

HLA-DR2 Tg mice bearing chimeric MHC class II molecules were developed as previously described.⁴⁰ The peptide-binding domain of MHC class II is encoded by human sequences while the membrane proximal portion including the CD4-binding domain is encoded by mouse sequences (DR α 1*0101: I-E α and DR β 1*1501: I-E β). The DR α 1*0101: I-E α construct was kindly provided by Dr Dennis M Zaller. The DR β 1*1501: I-E β construct was made essentially as described in Woods *et al.*⁴⁰ with the following changes. The pACYC184 vector containing the DRB1*0401 exons 1 and 2, and the E β ^d exons 3–6 was partially digested with BamHI and treated with Klenow polymerase to remove a BamHI site in the vector. Subsequently, DRB1*1501 exon 2 was cloned into pACYC184 which had been predigested with BamHI and EcoRI to remove DRB1*0401 exon 2. Transgenic mice were generated by microinjecting the chimeric α - and β -chain constructs into fertilized eggs from (DBA/2 $\times$ C57BL/6)F₁ matings. Viable embryos were transferred into pseudo pregnant females for development to term. Transgenic offspring were backcrossed twice to the MHC class II knock out mouse, MHCII Δ/Δ .⁴¹

Induction of active EAE and treatment with RTLs

Tg HLA-DR2 male and female mice between 8 and 12 weeks of age were immunized subcutaneously as described⁴² at four sites on the flanks with 0.2 cm³ of an emulsion comprised of 200 μ g mouse MOG-35-55 peptide in complete Freund's adjuvant (CFA) containing 400 μ g *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI). In addition, mice were given pertussis toxin (Ptx, List Biological Laboratories, Campbell, CA) on Day 0 and Day 2 post-immunization (25 ng and 67 ng per mouse, respectively). Mice were treated iv daily for 8 days, beginning 2–4 days after onset of clinical signs, with 100 mm³ of RTL312, RTL342, or vehicle (20 mmol dm⁻³ Tris, pH 8.5) containing 33 μ g of the RTL proteins. Actively immunized mice were assessed daily for clinical signs of EAE according to the following scale: 0 = normal; 1 = limp tail or mild hind limb weakness; 2 = limp tail and moderate hind limb weakness or mild ataxia; 3 = limp tail and moderately severe hind

limb weakness; 4 = limp tail and severe hind limb weakness or mild forelimb weakness or moderate ataxia; 5 = limp tail and paraplegia with no more than moderate forelimb weakness; and 6 = limp tail and paraplegia with severe forelimb weakness or severe ataxia or moribund condition. The average daily score was determined for each mouse by summing the daily clinical scores and dividing by the number of days the mouse exhibited clinical signs. The mean peak and average daily scores plus or minus SD were calculated for the control and experimental groups.

RESULTS

We have recently described protein engineering studies of recombinant TCR ligands (RTLs) derived from the α -1 and β -1 domains of HLA-DR2 (DRB1*1501/DRA*0101).²⁷ These molecules formed well defined aggregates that were highly soluble in aqueous buffers, with retention of biological activity.^{11,28,29} We analyzed the membrane proximal surface of the β -sheet platform that packed on the membrane distal surfaces of the α 2 and β 2 Ig-fold domains, specifically looking for features that might contribute to higher-order structures or aggregation (Plate 1). We grouped these residues, based on their location within the β -sheet platform and on their relative level of interaction with residues from the α 2 and β 2 Ig-fold domains, and constructed a series of site-directed mutants, replacing single and then multiple residues with either serine or aspartic acid residues. The study developed in two stages, with the first stage focused on obtaining soluble proteins that were monodisperse, and the second focused on biophysical and biochemical characterization of the modified molecules. Reiterative site-directed mutagenesis allowed us to generate two modified RTLs that were suitable for further biological characterization (Table 1).

RTL302 could be converted to a monomer with either five serine (5S) or five aspartate (5D) substitutions, RTL302(5S) and RTL302(5D), respectively, within a group of residues along the external face of the first strand of anti-parallel β -sheet derived from the α -chain of the HLA-DR2 progenitor molecule. We have termed these the group I core residues (Plate 1(C)). Comparison of the 5S or 5D modified molecules with RTL302 by size exclusion chromatography (SEC) (Fig 1(A)) demonstrated that both RTL302(5S) and RTL302(5D) behaved as approximately 25 kDa monomers. Dynamic light scattering (DLS) was used to measure the diffusion constants and calculate hydrodynamic radii for the molecules (Table 2), and these studies demonstrated unequivocally that RTL302(5S) and RTL302(5D) were monomeric. When Ag-peptides were covalently tethered to the amino-terminus of the molecules, their properties varied nominally depending on the Ag-peptide used, and more importantly, differed depending on the presence of the polar 5S or charged 5D modifications. Comparing RTL320 (5S modification, covalently tethered MBP-85-99 peptide) with RTL340 (5D modification, covalently tethered MBP-85-99 peptide), RTL320 still tended to aggregate, with a portion of the molecules (15%) formed into multimers. RTL340 was completely monomeric, and was more robust in terms of being able to accommodate various covalently tethered Ag-peptides such as MBP-85-99 (RTL340) and MOG-35-55 (RTL342) without significant alteration of the monomers solution properties (Fig 1; Table 2).

Further biochemical analysis demonstrated that the 5S- and 5D-modified molecules retained their native structure. RTLs contain a native conserved disulfide bond between cysteine 16 and 80 (RTL302 amino acid numbering, corresponding to HLA-DR2 β -chain residues 15 and 79). Air oxidation of these residues to reconstitute the native disulfide bond was demonstrated by a gel shift assay in which identical samples with or without the reducing agent β -mercaptoethanol (β -ME) were boiled 5 min prior to SDS-PAGE. In the absence of β -ME disulfide bonds are retained and proteins typically demonstrate a higher mobility during electrophoresis through acrylamide gels due to their more compact structure. All of

the RTL molecules produced showed this pattern, indicating the presence of the native conserved disulfide bond (data not shown). These data represent a primary confirmation of the conformational integrity of the molecules.

Circular dichroism (CD) demonstrated the highly ordered secondary structures of the RTL constructs. The RTLs without covalently tethered Ag-peptide contained 20–25% α -helix, 21–27% anti-parallel β -strand, and 20–22% β -turn structures (Fig 2(A); Table 3). The RTLs with covalently tethered Agpeptides contained 15–19% α -helix, 19–22% anti-parallel β -strand, and 18–23% β -turn structures (Fig 2(B); Table 3). These three basic secondary structures of a polypeptide chain (helix, sheet, coil) each show a characteristic CD spectrum in the far UV, and a protein consisting of these elements displays a spectrum that can be deconvoluted into each of the individual contributions. Although there are limitations inherent in the method (such as the lack of consideration of chromophore interaction(s) within different structural regions), the fit is quite acceptable for what would be expected for a qualitative assessment of the RTL protein fold and is consistent with our previous data collected for the multimeric versions of the RTLs.²⁷ The monodisperse monomeric RTLs retain the native structure of the progenitor Ag-binding/TCR recognition domain of HLA-DR2.

We also used CD to monitor structure loss upon thermal denaturation. The RTLs exhibited a high degree of thermal stability, and non-linear least-square analysis indicated that RTL303 and RTL320 are cooperatively folded (Fig 2(C)). The temperature (T_m) at which half of the structure was lost in 20 mmol dm⁻³ Tris, pH 8.5, was difficult to determine because of the high melting temperatures observed. Extrapolation of the curves using non-linear analysis yields a T_m of 92 °C for RTL303, 87 °C for RTL320. The melting temperature for RTL340 was extremely high, and since so little of the full transition can be observed a T_m could not be determined accurately. We had previously reported a T_m for RTL303 of 78 °C when the molecule was solubilized in PBS²⁷ reflecting the effect solvent had on the overall stability of the molecules.

We used a 'peptide capture' ELISA assay with biotinylated-MOG to compare Ag-peptide binding to RTL302, RTL302(5S), and RTL302(5D). Non-linear regression analysis using a one-site (hyperbola) binding model was used to calculate a B_{max} and K_d for the molecules (Fig 3(A)). These values are indicators of relative affinities, and should not be taken as the absolute values. As shown in Fig 3(B), binding of MOG peptide (0.15 μ mol dm⁻³) to RTLs as a function of time was extremely fast. Using linear regression analysis the initial rate of MOG binding was calculated to be $0.17 \pm 0.06 \Delta OD \text{ min}^{-1}$ for RTL302, $0.11 \pm 0.02 \Delta OD \text{ min}^{-1}$ for RTL302(5S), and 0.10 ± 0.02 for RTL302(5D). While the calculated B_{max} remained virtually unchanged for all three molecules, the 5S and 5D versions of RTL302 showed increased K_d values (Fig 3, insert). RTL302 had a K_d of $0.027 \mu\text{mol L}^{-1}$, whereas RTL302(5S) and RTL302(5D) had K_d values of $0.043 \mu\text{mol L}^{-1}$ and $0.049 \mu\text{mol L}^{-1}$, respectively. Because of this difference in their equilibrium dissociation constants, we sought to characterize the biological efficacy of the altered RTLs containing covalently tethered MOG-peptide.

We characterized the *in vitro* activity of the RTLs in an assay designed to quantify their ability to induce Ag-specific inhibition of T cell proliferation.^{11,12,29} The DR2-restricted T cell clone 4-G1 is specific for the MBP-85-99 peptide. Cells that were not pretreated with RTLs ('untreated' control) showed a 68 $\times$ stimulation index and cells pretreated with 'empty' RTL302 showed close to 90 $\times$ stimulation index, a 31% increase above the 'untreated' control. Pre-incubation with RTL303, RTL320 or RTL340 all showed greater than 90% inhibition of proliferation compared with the 'untreated' control (Table 4).

MOG-35-55-induced experimental autoimmune encephalomyelitis (EAE) in DR2 (DRB1*1501) transgenic (Tg) mice serves as an extremely useful animal model of multiple sclerosis (MS)²⁶ characterized by a moderately severe chronic disease with 100% penetrance. Characteristics of the disease include ascending paralysis marked by inflammatory, demyelinating CNS lesions. EAE was induced with MOG-35-55 peptide/CFA on day 0 plus Ptx on days 0 and 2, and initial symptoms of disease can be observed beginning about 10 days after induction. To evaluate the clinical potential of the monomeric RTL342, we treated Tg-DR2 mice with MOG-induced EAE 2–4 days after onset of clinical signs with RTL312, RTL342, or vehicle alone (Fig 4; Table 5). Treatment with RTL312 or RTL342 rapidly reversed established clinical signs of EAE (score about 2.5) to an average daily score of <0.5 units by the end of the eight-day treatment period. This low degree of disability was maintained without further RTL injections over the remainder of the observation period, which in one experiment lasted for 5 weeks after treatment was stopped. In contrast to the reversal of EAE mediated by RTL312 or RTL342, control groups receiving vehicle or 33 µg per injection of non-Ag-specific RTL303 (containing MBP-85-99 peptide; data not shown) developed moderately severe chronic EAE (score of >4).

DISCUSSION

Our results demonstrated that potent biological activity of DR2-derived RTLs^{11,12,29} was retained when these molecules are produced in a monomeric form. The biological activity retained included Ag-peptide binding and the ability to inhibit T cell proliferation *in vitro*. The latter is extremely important from a clinical perspective. Monomeric RTL342 was able to reverse clinical disease and induced long-term T cell tolerance against the encephalitogenic, DR2-restricted, MOG-35-55 peptide in Tg mice. These mice uniquely express this multiple sclerosis-associated HLA-DR2 allele. Our earlier studies had demonstrated that immunization of Tg-DR2 mice with MOG-35-55 peptide induced strong T cell responses, perivascular spinal cord lesions with demyelination, and severe chronic signs of EAE, as well as anti-MOG antibodies that were apparently not involved in either disease or tolerance induction.²⁹ Treatment of the Tg-DR2 mice after onset of clinical EAE with an 8-day course of daily iv injections of 33 µg RTL342 reversed disease progression to baseline levels and maintained reduced clinical activity even after cessation of further injections. Treatment with control RTL303 containing covalently tethered MBP-87-99 did not inhibit EAE or affect T cell responses to MOG-35-55 peptide, demonstrating antigen specificity. The potent clinical activity of the monomeric RTLs coupled with the solution-phase properties documented here have allowed us to proceed with intensive evaluation of these molecules for therapeutic intervention in human clinical trials for treatment of multiple sclerosis.

The peptide binding/TCR recognition domain of MHC class II from which RTLs are derived contain a complex mixture of α -helix and β -sheet secondary structure, as well as a highly conserved post-translational modification, a disulfide bond between cysteines at position 16 and 80 (RTL302 numbering). The molecules are small enough to systematically dissect with currently available technology yet complex enough that it is a fair assumption that any design concepts learned (see below) will be applicable to other proteins. The domain is derived from two separate polypeptide chains, and the MHC genes HLA-DR, HLA-DQ, and HLA-DP are the most polymorphic genes in the human genome.

MHC class II molecules have (at least) three clearly defined biochemical 'functions' that can be used to evaluate and quantify the retention of a specific three-dimensional fold derived from the primary sequence: Ag-peptide binding, TCR binding and CD4 binding. We hypothesize that these functions have been encoded and superimposed onto the primary sequence of MHC class II, and that some of these functions can be separated experimentally

for evaluation using protein engineering.^{26,27} The two key biochemical functions we sought to retain were the ability to specifically bind Ag-peptides and the ability to bind the $\alpha\beta$ heterodimer chains of the TCR. Retention of these key features should allow us to discern the minimal interaction interface with the T cell that still initiates a throughput information signal,¹² allowing us to engineer a molecular system for controlling CD4⁺ T cells in an Ag-specific manner.

While HLA-DR2-derived RTLs with the wild-type sequence retained these two key biological activities,²⁹ they tended to form higher order structures¹¹ that could not be completely eliminated by manipulating solvent conditions. For example, we found that by decreasing the concentration of purified RTL302 protein to 0.1 mg cm⁻³ for the final folding step, and changing buffers from phosphate-buffered saline to Tris, we could increase the yield of monodisperse monomeric RTL302 to almost 20%. However, concentrating purified RTL302 monomer above 0.2 mg cm⁻³ caused the molecules to repartition back into a mixture of monomer and aggregate, an equilibrium that was concentration dependent. Our recent success in producing monomeric HLA-DP- and murine I-A^s derived RTLs that retained biological activity (Meza-Romero, personal communication; and Ref 43) led us to propose that the aggregation of HLA-DR2 derived RTLs was specific to certain portions of the DR2-derived RTL sequence.

With the availability of the 2.6 angstrom resolution crystal structure of HLA-DR2 with bound Ag-peptide MBP-85-99 (PDB accession 1BX2; (30)), we analyzed the membrane proximal surface of the β -sheet platform and the membrane distal surfaces of the $\alpha 2$ and $\beta 2$ Ig-fold domains, specifically looking for features that contributed to higher-order structures or aggregation. The β -sheet platform that was buried in the progenitor HLA-DR2 molecule now defined the external face of the RTLs distal from the peptide binding groove. This surface contained a number of hydrophobic residues typically found buried within a protein structure rather than being solvent exposed. The propensity of different amino acid residues to be present in β -sheet structures has been intensively investigated,⁴⁴⁻⁴⁹ as part of an overall goal of understanding the rules that dictate secondary structure stability and formation. The body of work available has defined the markedly high preference in β -sheets for the β -branched amino acids isoleucine, valine, and threonine, as well as aromatic amino acid residues phenylalanine and tyrosine. Replacement of hydrophobic residues with polar serine and charged aspartate residues (Plate 1), meant that we were able to modify the RTL surface to be much less prone to aggregation. While aspartate is significantly under represented in β -sheet regions of proteins, in this study we show that the introduction of five serine or five aspartate residues on the external face of an interior strand of the β -sheet platform of RTLs has only a subtle effect on the secondary structure as quantified by circular dichroism (Fig 2), interpreted as an approximately 10% increase of anti-parallel β -strand structure upon deconvolution of the spectra (Table 3).

An explanation for why the modified RTLs maintain the same basic fold and retain their biological features comes from analysis of the β -sheet in the context of its functional role as an 'open' platform in the overall tertiary structure of the Ag-binding/TCR recognition domain of the progenitor HLA-DR2 molecule, rather than a closed surface like an Ig-fold. The β -strand that we successfully modified here defines an extended, central strand within the progenitor HLA-DR2 molecule (Plate 2). Introduction of aspartate residues may force the molecule to maintain an extended surface, 'stretched' out along this interior β -strand by charge-charge repulsion, rather than allowing the structure to collapse onto itself in the absence of the Ig-fold domains that are present in the progenitor HLA-DR2 molecule. Our data support the idea that β -sheet propensity for an amino acid residue arises from local steric interactions of the amino acid side chain with its local backbone.⁴⁴ The studies presented here demonstrated that RTLs encoding the Ag-binding/TCR recognition domain

of MHC class II molecules are innately very robust structures, capable of retaining activity separate from the Ig-fold domains of the progenitor class II structure, and even after fairly aggressive modification to make the molecules monomeric and monodisperse. Increased solubility and prevention of aggregation has been accomplished by modification of an exposed surface that was buried in the progenitor structure. By staying within thermodynamic limitations that constrain the proteins' final folded structure, and by not interfering with the process by which the protein domain achieves this final fold, the first major hurdle involved in recombinant design of the RTLs appears to be accomplished, that of leaving intact within the primary sequence the 'ensemble code' that drives folding toward a final unique structure. The re-designed RTLs bind peptides and the TCR in an Ag-peptide-specific manner, with retention of potent biological activity.

Acknowledgments

This work was supported by the National Institutes of Health grants AI43960, ES10554 and NS41965 (to GGB), The National Multiple Sclerosis Society grant RG3012A (to GGB), the Department of Veterans Affairs (to AAV) and the Nancy Davis Center without Walls (to GGB).

Contract/grant sponsor: National Institutes of Health; contract/grant number: AI43960

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NOTATION

Ag	Antigen
APC	Antigen-presenting cell
β-ME	β-Mercaptoethanol
CD	Circular dichroism
CFA	Complete Freund's adjuvant
DLS	Dynamic light scattering
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbant assay
HLA	Human leukocyte antigen
hu-	Human; MBP, Myelin basic protein
MHC	Major histocompatibility complex MOG Myelin oligodendrocyte glycoprotein (murine sequence)
MS	Multiple sclerosis
NFDM	Non-fat dry milk
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
Ptx	Pertussis toxin
RPMI	Growth media for cells developed at Roswell Park Memorial Institute
RT	Room temperature
RTL	Recombinant T cell receptor ligand
sc	Subcutaneous
SEC	Size exclusion chromatography
STR-HRP	Streptavidin–horseradish peroxidase conjugate
TCR	T cell receptor
Tg	Transgenic

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Figure 1.

Size exclusion chromatography of modified RTLs. Purified and refolded RTLs were analyzed by size exclusion chromatography (SEC). (A) SEC of RTL302 (black), RTL302(5S) (red) and RTL302(5D) (green). These RTLs do not contain covalently tethered Ag-peptides. (B) SEC of RTLs derived from the wild-type HLA-DR2 containing covalently tethered Ag-peptide MBP-85-299 (RTL303, black) or MOG-35-55 (RTL312, yellow). The 5S and 5D variants of RTL303 (RTL320 red, and RTL340 green, respectively) and the 5D variant of RTL312 (RTL342, blue) are also displayed. The Superdex 75 16/60 size exclusion column was calibrated with a set of proteins of known molecular weight with exclusion volumes as indicated by *; myoglobin, 17.3 kDa; ovalbumin, 43 kDa; bovine serum albumin 67 kDa; catalase 232 kDa; thyroglobulin, 670 kDa.

Figure 2.

Circular dichroism (CD) spectra of modified DR2-derived RTLs. (A) CD spectra of 'empty' RTL302 (black), RTL302(5S) (red) and RTL302(5D) (green). (B) CD spectra of RTLs containing covalently tethered Ag MBP-85-99 peptide. RTL303, (black), RTL320 (red), and RTL340 (green). (C) Thermal denaturation curves for RTL303, RTL320 and RTL340 show a high degree of cooperativity and stability. RTL340 was resistant to complete thermal denaturation and aggregation and is soluble even after boiling for 5 min. Unless otherwise indicated, CD measurements were performed at 25 $^{\circ}\text{C}$ on an Aviv-215 instrument using 0.1 mm cell from 260 to 180 nm on protein samples in 20 mmol dm^{-3} Tris-Cl, pH 8.5. Concentration of each protein was determined by amino acid analysis. Data are expressed as $\Delta\epsilon$ per mole per cm. Analysis of the secondary structure was performed using the variable selection method.³⁹

Figure 3.

Direct measurement of peptide binding to HLA-DR2-derived RTLs. Binding of biotinylated-MOG to RTL302, RTL302(5S), and RTL302(5D). (A) Saturation as a function of biotinylated-MOG concentration. Insert: Scatchard analysis of peptide binding. (B) Binding of biotinylated-MOG peptide ($0.15 \mu\text{mol dm}^{-3}$) to RTLs as a function of time to compare the initial rate of binding.

Figure 4.

Monomeric, monodisperse RTL342 was as effective as RTL312 at treating EAE in DR*1501 transgenic animals. Mean clinical scores of HLA DR2 (DRB1*1501/DRA*0101) transgenic mice treated with 33 μ g of RTL312, RTL342, or vehicle alone (Tris, pH 8.5). All mice were immunized sc with 200 μ g MOG-35-55 and 400 μ g CFA in conjunction with 100 ng Ptx iv on Day 0 and 266 ng Ptx 2 days post-immunization. On Day 14 all mice were distributed into six groups according to similarity in disease and gender. Mice were iv injected daily with RTL312, RTL342, or vehicle (n 4 per group, except for vehicle group where n = 3; arrows indicate treatment).

Plate 1.

HLA-DR2, RTL302, and the solvent accessible surface of the RTL β -sheet platform. (A) Scale model of an MHC class II molecule on the surface of an APC and (B) RTL302, the soluble single-chain molecule derived from the antigen-binding/T cell recognition domains. The structures are based on the crystallographic coordinates of HLA-DR2 (PDB accession code 1BX2), and the transmembrane domains are shown schematically as 0.5 nm cylinders. Color scheme: α -chain, red; β -chain, blue. Bound antigenic peptide is green. The amino and carboxyl termini of HLA-DR2 and RTL302 are labeled N, C, respectively. Disulfide bonds are displayed as ball and stick models. (C) The hydrophobic residues of the β -sheet platform of RTL302. β -sheet strands are depicted in ribbon form (yellow) and the hydrophobic residues are colored according to three groups based on their location within the β -sheet platform and on their relative level of interaction with residues from the α 2 and β 2 Ig-fold domains. Group I residues V102, I104, A106, F108, L110 (red) comprised a central core along β -strand 1 of the α -1 domain, and, peripheral to this core, L9 and M119 (blue). Group II residues F19, L28, F32, V45, and V51 (green) were β -1 domain residues and group III residues A133, V138 and L141 (green) were from the α -1 domain.

Plate 2.

Interaction surface between the $\alpha 1\beta 1$ peptide binding/T cell recognition domain and the $\alpha 2\beta 2$ -Ig-fold domains of HLA-DR2. The interaction surface between the $\alpha 1\beta 1$ peptide binding/T cell recognition domain and the $\alpha 2\beta 2$ -Ig-fold domains was modeled and refined using the high resolution human class II DR2 structure 1BX2.³⁰ The transmembrane domains are shown schematically as 0.5 nm cylinders. Color scheme: α -chain, red; β -chain, blue. The amino and carboxyl termini of MHC class II are labeled N, C, respectively. Cysteines are rendered as ball-and-stick, as are the five residues V102, I104, A106, F108, L110 (1BX2 numbering). The interaction surface (4 angstrom interface) between the Ig-fold domains and the peptide binding/T cell recognition domain is colored by lipophilic potential (LP). Water molecules within this interface in the 1BX2 crystal structure are shown as red spheres.

Table 1

Molecules used in this study

Molecule	Description
RTL302	Human HLA DR2(DRB1* 150101/DRA*0101) β 1 α 1 domains
RTL302 (5S)	RTL302 (V102S, I104S, A106S, F108S, L110S) ^a
RTL302 (5D)	RTL302 (V102D, I104D, A106D, F108D, L110D)
RTL303	RTL302/MBP-85-99 ^b
RTL312	RTL302/MOG-35-55 ^c
RTL320	RTL303 (5S)
RTL340	RTL303 (5D)
RTL342	RTL312(5D)

^aRTL302 numbering. These residues correspond to HLA-DR2 α chain residues V6, 18, A10, F12, and L14. Residue numbering is increased in the Ag-tethered molecules to account for the Ag-peptide (variable length) plus linker (15 residues). ^d RTL302 and derivatives do not contain covalently tethered Ag-peptide; RTL303-342 do contain covalently tethered Ag-peptides.

^b MBP-85-99, ENPWHFFKNIVTPR.

^c MOG-35-55, MEVGWYRSPFSRWHLRNGK.

^d The 15-residue linker (GGGSLVPRGSGGGG) containing an embedded thrombin cleavage site that was used to tether the covalently coupled Ag-peptides has been previously described.^{17,27}

Table 2

Hydrodynamic analysis of RTLs by dynamic light scattering

Molecule	Radius (nm)	Estimated MW(kDa)
RTL302 (peak I) ^a	17.6	2760
RTL302 (peak II)	2.5	27
RTL302 (5S)	2.5	27
RTL302 (5D)	2.3	25
RTL303	15.4	2030
RTL312(peak I)	15.2	1970
RTL312(peak II)	4.3	102
RTL320 (peak I)	13.5	1490
RTL320 (peak II)	4.8	131
RTL340	2.5	28
RTL342	2.6	31

Hydrodynamic status of modified RTLs were analyzed by light scattering analysis using a DynaPro™ molecular sizing instrument (Protein Solutions, Inc).

^aSome of the proteins showed two clearly defined peaks by SEC and these were characterized independently. Peak I refers to the aggregate (larger) peak, and peak II refers to the smaller size, in most cases monomeric fraction.

Table 3

Secondary structure analysis of RTLs

Molecule	α -Helix	α -Parallel β -Sheet	Parallel β -Sheet	β -turn	Random coil	Total
RTL302 (peak I)	0.21	0.21	0.02	0.23	0.33	0.99
RTL302 (peak II)	0.20	0.27	0.00	0.20	0.32	0.99
RTL302(5S)	0.20	0.21	0.02	0.22	0.34	1.00
RTL302(5D)	0.20	0.27	0.00	0.20	0.20	1.00
RTL303	0.26	0.20	0.04	0.19	0.32	1.00
RTL312	0.18	0.24	0.07	0.17	0.31	0.96
RTL320 (peak I)	0.22	0.22	0.03	0.21	0.32	1.00
RTL320 (peak II)	0.19	0.19	0.03	0.23	0.35	1.00
RTL340	0.15	0.20	0.03	0.27	0.35	1.00
RTL342	0.19	0.22	0.05	0.18	0.30	0.93

Secondary structure content derived from the deconvoluted spectra of the RTLs (see text).

Table 4

Antigen-specific inhibition of T cell proliferation by pre-mcubating with RTLs

Clone EN4-G1	Pre-incubation				
	Untreated	RTL302	RTL303	RTL320	RTL340
+APC alone	588.97	569.1	578.7	592.0	641.9
+APC/MBP-85-99 (10 μ g cm ⁻³)	40144.67	50841.1	2560.4	1847.7	1515.8
Stimulation Index	68	89	4	3	2
Inhibition (%)	–	+31.1	–93.5	–95.2	–96.5

Each data point represents the average of triple wells from each treatment.

Table 5

RTL treatment of DR2 transgenic mice

Treatment	Incidence	Onset	Peak	Mortality	CDI
RTL312	4/4	9.5 ± 2.8	3 ± 1.8	0/4	29.8 ± 21.7 ^a
RTL342	4/4	10.8 ± 2.2	2.6 ± 1.1	0/4	16 ± 10.9 ^a
Vehicle	3/3	12.3 ± 1.2	6 ± 0	0/4	133.7 ± 11.1

^aSignificant difference between experimental group and vehicle group (p < 0.001).