

The central residues of a T cell receptor sequence motif are key determinants of autoantigen recognition in murine experimental autoimmune encephalomyelitis

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The autoreactive response in murine experimental autoimmune encephalomyelitis (EAE) is dominated by an oligoclonal expansion of V β 8⁺ CD4⁺ T cells. These T cells recognize the immunodominant N-terminal nonapeptide of myelin basic protein (MBP1–9) associated with the MHC class II molecule, I-A^u. Amongst the autoreactive cells, T cells bearing TCR containing the CDR3 β motif Asp-Ala-Gly-Gly-Gly-Tyr (DAGGGY) play a dominant role in the disease process. Here we have investigated the molecular basis for antigen recognition by a representative TCR (172.10) that contains the DAGGGY motif. The roles of the three glycines in this motif in the corresponding TCR-peptide-MHC interactions have been analyzed using a combination of site-directed mutagenesis and surface plasmon resonance. Our data show that mutation of either of the first two glycines (G97, G98) to alanine results in soluble, recombinant TCR that do not bind to recombinant antigen at detectable levels. Mutation of the third glycine (G99) of the 172.10 TCR results in a substantial decrease in affinity. The importance of the triple glycines for antigen recognition provides an explanation at the molecular level for the recruitment of T cells bearing the DAGGGY motif into the responding repertoire during EAE induction.

Received 20/7/04

Revised 24/9/04

Accepted 20/10/04

[DOI 10.1002/eji.200425501]

Key words:

Experimental autoimmune encephalomyelitis · Surface plasmon resonance · Pathogenic clones · T cell recognition · Affinity

Introduction

Murine EAE in H-2^u mice is characterized by an oligoclonal expansion of pathogenic, CD4⁺ T cells [1, 2]. These T cells recognize the immunodominant, N-terminal epitope of myelin basic protein (MBP1–9, acetylated at position 1) associated with the MHC class II molecule, I-A^u [3]. The antigen-specific T cells almost

invariably express V β 8, with a less marked bias towards Va2 or Va4 usage [1, 2]. Recent studies involving immunoscope analysis have shown that in B10.PL (H-2^u) mice, TCR expressing V β 8.2-J β 2.6 with a conserved CDR3 motif (Asp-Ala-Gly-Gly-Gly-Tyr or DAGGGY) are expressed on Th1-type clones that dominate the encephalitogenic repertoire during actively induced disease [4]. Such encephalitogenic clones appear to play an essential role in disease induction [4–6].

A β chain comprising V β 8.2-J β 2.6 with the DAGGGY motif in CDR3, in combination with a Va2.3-Ja39 chain, constitutes the TCR expressed by the well-characterized 172.10 T cells [7, 8]. Transgenic mice expressing this TCR are susceptible to spontaneous EAE, demonstrating the encephalitogenic potential of 172.10 T cells [7]. The kinetics and affinity of the interaction of recombinant, soluble 172.10 TCR with cognate ligand (MBP1–9 complexed with I-A^u) have been characterized using

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Abbreviations: CDR: Complementarity determining region ·

EAE: Experimental autoimmune encephalomyelitis ·

MBP: Myelin basic protein · pMHC: Peptide-MHC · scTCR: Single chain TCR · SPR: Surface plasmon resonance

surface plasmon resonance (SPR) [9]. The TCR has a higher affinity than other EAE-associated TCR in H-2^u mice such as the 1934.4 TCR that comprise V α 4 in combination with V β 8.2–J β 2.3 or V β 8.2–J β 2.4 containing DASGAE or DASGGN motifs in the third CDR [4, 9]. As CDR3 residues of TCR frequently play a major role in antigen recognition (e.g. [10–15]), it is therefore possible that the DAGGGY motif might contribute to the higher affinity of 172.10-like T cells.

In addition to providing affinity data, analyses of the temperature dependence of TCR-peptide-MHC (pMHC) binding by SPR can be used to estimate the entropy, enthalpy and heat capacity changes associated with such interactions [9, 16, 17]. From studies in multiple systems, unfavorable (negative) entropic terms appear to be a general feature of TCR-pMHC interactions [9, 16–19], consistent with the concept that TCR undergo significant conformational rearrangements during antigen recognition [11]. Plasticity of TCR has been suggested [11, 16, 17] to contribute to a characteristic and apparently essential feature of TCR, namely their high degree of cross-reactivity [6, 20–24]. In this context, thermodynamic studies of 172.10 TCR-pMHC binding demonstrate that in addition to its higher affinity, this interaction is more entropically unfavorable than the 1934.4 TCR-pMHC interaction [9]. This suggests that the 172.10 TCR is more flexible and undergoes a greater extent of remodeling during antigen binding [9], consistent with its higher degree of cross-reactivity relative to 1934.4 cells [20, 25]. The triple glycine motif within the DAGGGY sequence would be predicted to be flexible [9], and this sequence might therefore contribute to the entropic properties of the 172.10 TCR.

Although multiple predisposing factors interact to induce autoimmune disease (reviewed in [26]), data in both H-2^u mice and other autoimmune disease models support the concept that the avidity of autoreactive T cells may be a contributing factor towards the onset and progression of disease [4, 27–30]. Given the dominance of the DAGGGY motif in the response to MBP1–9:I-A^u [4, 5], it is of interest to investigate the molecular nature of antigen recognition by representative TCR that contain this sequence. We have therefore mutated the three glycine residues of the DAGGGY motif of the 172.10 TCR to alanine, and compared the interaction properties of the mutated TCR with those of the parent, wild-type TCR. The studies demonstrate that two of the three glycines are essential for antigen binding, whereas the third glycine in the motif plays a lesser, but significant, role. Our observations provide a rationale at the molecular level for the efficient recruitment of T cells bearing TCR with the DAGGGY motif into the responding repertoire following disease induction.

Results and discussion

The central glycines play an important role in ligand recognition

Recombinant, mutated 172.10 single chain TCR (scTCR; G97A, G98A and G99A, with nomenclature G97A indicating glycine 97 to alanine etc.) were refolded in similar yields and used in comparative binding analyses with the wild-type 172.10 scTCR using SPR. Fig. 1 shows a trace of the G98A mutant during the final gel filtration chromatography step. Similar profiles were observed for the wild-type and other two mutated TCR. The SPR studies were carried out with recombinant MBP1–9[4Y]:I-A^u [31] immobilized on the sensor chip. The data show that all mutations result in considerable losses in binding activity (Fig. 2). For both G97A and G98A, the essentially negligible binding signal at a concentration of ~20–40 μ M precludes determination of dissociation constants (Fig. 2). These scTCR were therefore not analyzed further. For G99A, a less marked loss in affinity was seen, and the equilibrium dissociation constant was estimated to be 83.3 μ M (Fig. 2D). Thus, the G99A mutation results in a substantial loss in affinity relative to the wild-type 172.10 scTCR (8.8 μ M [9]; this study). Affinity analyses were carried out with three different preparations of G99A and in all cases a similar loss in affinity relative to the wild-type 172.10 scTCR was observed (data not shown). Dissociation constants in the range of that for the G99A TCR are technically demanding to determine, and our main goal here was to ascertain whether the G99A TCR resulted in a reduction of affinity relative to the wild-type TCR, rather than to obtain an absolute value for the K_D . Comparative analyses of the binding signal (Req) for the

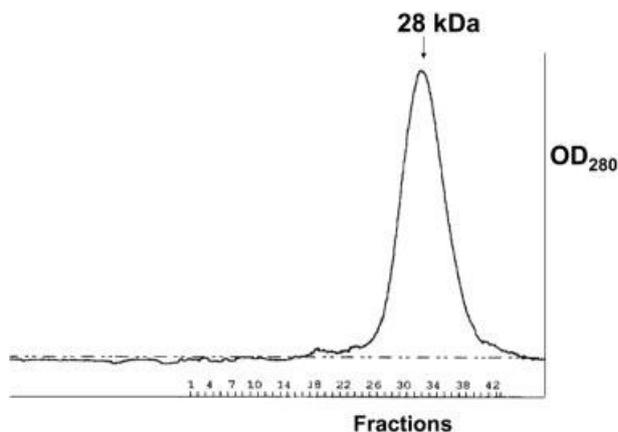


Fig. 1. Gel filtration chromatography of refolded 172.10 scTCR (G98A mutant) using a HiLoad 26/60 SuperdexTM preparative grade column following refolding. The arrow indicates the mobility of a protein standard of molecular mass 28 kDa (molecular mass of scTCR is 25.6 kDa).

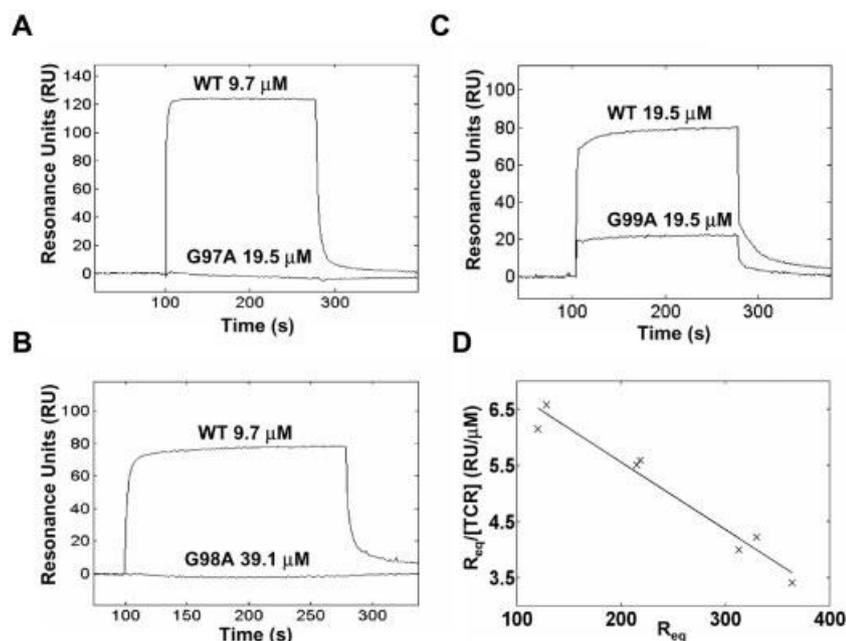


Fig. 2. Comparative SPR analyses of the binding of G97A (A), G98A (B) and G99A (C) with wild-type 172.10 scTCR. Wild-type and mutated, refolded scTCR were injected over flow cells coupled with MBP1–9[4Y]:I-A^u [to coupling densities of 877 RU (A) or 900 RU (B, C)] at a flow rate of 10 μ l/min. Concentrations of proteins used are indicated. Data were zero adjusted and reference cell subtracted. Sensorgrams shown are representative of duplicate injections. (D) Equilibrium binding analyses of the G99A-scTCR interaction. G99A scTCR was injected in concentrations ranging from 19–105 μ M over a flow cell coupled with MBP1–9[4Y]:I-A^u (coupling density of 1,900 RU) at a flow rate of 10 μ l/min. Data were zero adjusted, reference cell subtracted and R_{eq} values used to generate a Scatchard plot. Similar results were obtained using lower coupling densities of MBP1–9[4Y]:I-A^u.

G99A and wild-type TCR were consistent with the affinity difference (e.g. Fig. 2C). The dissociation of G99A scTCR from ligand was too rapid to allow an accurate determination of the off-rate, precluding K_D estimation from kinetic parameters (Fig. 2).

The thermodynamics of the interaction of the G99A mutant

The thermodynamics of TCR-pMHC interactions can be estimated by analyzing the effect of temperature on the interaction affinity and van't Hoff analyses [9, 16, 17]. As the G99A mutant retained significant binding activity, we therefore determined the dissociation constants of this scTCR for immobilized MBP1–9[4Y]:I-A^u complexes at different temperatures ranging from 10 to 28°C (in 3°C increments) (Fig. 3). Our goal was to compare this mutated scTCR with the wild-type 172.10 scTCR, and we used a similar temperature range to that described previously for the wild-type receptor [9]. As the temperature increases, the on- and off-rates of the G99A-pMHC interactions increase (Fig. 3). This is similar to the observations for both the wild-type 172.10 TCR-pMHC interaction and TCR-pMHC interactions in distinct antigen recognition systems [9, 16, 17]. The off-rates at temperatures above about 19°C are immeasurably fast (Fig. 3). We therefore derived

'equilibrium' rather than 'kinetic' binding affinities from the sensorgrams, and used these to calculate the free energy change for the interaction at each temperature. ΔG° vs. temperature plots for this mutated TCR are shown in Fig. 3. Estimates for ΔH° , ΔS° and ΔC_p° values for the interaction of the G99A scTCR with MBP1–9[4Y]:I-A^u were derived from these plots using the approach described in the Materials and methods, and are shown in Table 1. The thermodynamics of the interaction of the wild-type 172.10 scTCR with MBP1–9[4Y]:I-A^u have been analyzed previously [9] and for comparative purposes the results from this analysis are also shown in Table 1 (in the current study, analyses of the wild-type 172.10 scTCR led to similar temperature dependencies: data not shown).

The results indicate that the temperature dependence of the G99A scTCR-pMHC interaction is very similar to that corresponding to the wild-type scTCR (Table 1, Fig. 3 and [9]). The lower affinity of the G99A scTCR for ligand is a result of a less favorable enthalpic term relative to that for the wild-type scTCR. The entropic and heat capacity terms are similar for both wild-type and mutated scTCR (Table 1). The ΔG° , ΔH° and $T\Delta S^\circ$ values for the G99A scTCR fall within the range reported previously in studies of other TCR-pMHC systems [9, 16–19]. However, it is noticeable that the heat capacity change (ΔC_p°) which is taken to be an

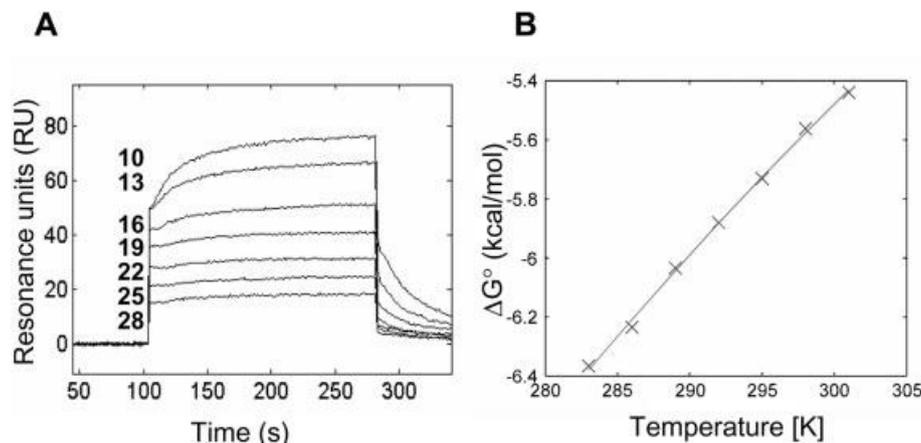


Fig. 3. Effect of temperature on the G99A scTCR-pMHC interaction. (A) G99A scTCR was injected at a concentration of 19.5 μM over a flow cell coupled with MBP1-9[4Y]:I-A^u (coupling density of 872 RU) at temperatures ranging from 10–28°C as indicated. Data were zero adjusted and reference cell subtracted. The majority of sensorgrams shown are representative of duplicate injections. (B) ΔG° vs. temperature plot for the data shown in Fig. 3A. The plot was fitted as in [9, 16] to derive the thermodynamic parameters shown in Table 1.

indication of the extent of burial of hydrophobic surface area upon complex formation [32] is numerically smaller than those values reported for other TCR-pMHC interactions [9, 16, 19]. The heat capacity change of -159 cal/mol.deg for the 172.10 scTCR-pMHC interaction is in the same range as the value of -250 cal/mol.deg predicted from average buried surface areas of crystallographically solved TCR-pMHC complexes [16]. The numerically low heat capacity change for the 172.10 TCR-pMHC interaction therefore distinguishes this TCR from others characterized to date [9, 16, 19], and suggests that there are outliers to the concept that TCR-pMHC interactions have unexpectedly large, negative ΔC_p° values.

In conclusion, the SPR data clearly show that the three glycines of the CDR3 β DAGGGY motif of the 172.10 TCR play a pivotal role in antigen recognition. Mutation of either of the first two glycines results in scTCR that do not interact at detectable levels with pMHC ligand in SPR experiments. The third glycine (G99) of this CDR plays a less marked, but still significant role. Whether the effects of mutating these glycines to alanine are direct, or due to some longer range conformational perturbation, cannot be ascertained from the current study. Our observations relate to the molecular basis of T cell recognition in murine EAE. In both EAE and other autoimmune disease models, higher avidity T cell clones have been suggested to play a central role in disease onset and progression [4, 27–30].

In EAE in H-2^u mice, the presence of T cells that are represented by the 172.10 clone, which has a relatively high avidity for antigen [9], correlates with disease activity [4, 5]. These clones are distinguished from other lower affinity clones by variations in CDR3 β [4]. Our analysis showing that the triple glycine motif in CDR3 β makes an important contribution to antigen recognition provides a rationale at the molecular level for the dominance of the DAGGGY motif in such encephalitogenic clones during actively induced EAE.

Materials and methods

Expression plasmids

The wild-type and mutated 172.10 scTCR (V α domain linked to V β domain by a 12-residue synthetic peptide linker) were expressed using an analogous expression construct to that described for periplasmic secretion of the 1934.4 TCR [33]. All plasmids contained the 'solubilizing mutations' of Ile75 to threonine and Leu78 to serine in the V β domain described in [9]. Mutations of Gly97, Gly98 and Gly99 individually to alanine were inserted using mutagenic oligonucleotides and standard methods of molecular biology.

Expression and purification of recombinant proteins

Recombinant proteins were expressed and purified from osmotic shock fractions of induced *E. coli* cultures using Ni²⁺-

Table 1. Thermodynamic parameters for the TCR-pMHC interactions

TCR	ΔG° (kcal/mol)	ΔH° (kcal/mol)	T ΔS° (kcal/mol)	ΔC_p° (cal/mol.deg)
Wild type	-6.9	-21.2	-14.3	-159
G99A	-5.6	-20.3	-14.7	-162

NTA-agarose as described in [33]. Purified protein was denatured and refolded as in [9] with the following modifications: the denatured scTCR (in 8 M urea) was diluted into 50 ml 2 M urea plus 2 mM glutathione/0.2 mM glutathione disulfide over a period of 6 h. The mix was then diluted by drop-wise addition of 250 ml 50 mM Tris-HCl, pH 8.0 over 6 h and then refolded for a further 36 h. Following refolding, monomeric scTCR were isolated using Ni²⁺-NTA-agarose followed by a HiLoad 26/60 Superdex prep grade column (Pharmacia).

The production of recombinant pMHC complexes (MBP1-9[4Y]:I-A^u), comprising the N-terminal nonapeptide of MBP covalently tethered to I-A^u (via the I-A^uβ chain) has been described previously [9, 31]. In this construct, N-terminal acetylation of the MBP is replaced by an N-terminal glycine, which retains T cell recognition [27, 31]. The MBP1-9 peptide in this construct has a tyrosine at position 4, which increases the affinity of the peptide for I-A^u [34–36] without affecting T cell recognition [37, 38].

Surface plasmon resonance studies

Analyses of the interactions of soluble TCR with immobilized, recombinant MBP1-9[4Y]:I-A^u complexes were carried out as in [9]. Recombinant MBP1-9[4Y]:I-A^u was site specifically biotinylated [31] and immobilized via streptavidin. As reference cells, flow cells of sensor chips were coupled with streptavidin only. In SPR experiments, two flow cells coupled with MBP1-9[4Y]:I-A^u and two with streptavidin (reference) were used to ensure reliability of data. The equilibrium dissociation constants (K_D) for the interactions of TCR with pMHC were assessed by injecting scTCR (~10–100 μM) in either 50 mM Tris-Cl pH 8.0/ 150 mM NaCl/0.01% Tween or HBS buffer (BIAcore), using a flow rate of 10 μl/min at a temperature of 25°C. The affinity of the G99A scTCR-pMHC interaction was determined by Scatchard analysis. The consistency of the affinity differences between wild-type and G99A scTCR for binding to pMHC was also analyzed by comparing equilibrium binding level signals (R_{eq}) for each of these scTCR at known concentrations, using the equation R_{eq} = (R_{max} [TCR])/(K_D + [TCR]), where R_{eq} = signal level at equilibrium (in resonance units, RU), R_{max} = maximal binding capacity of the flow cell, [TCR] = concentration of TCR, and K_D = dissociation constant.

To assess the effect of temperature on the K_D, equilibrium binding levels (R_{eq}) for fixed (non-saturating) concentrations of scTCR were determined as described in [9]. These values were used to derive K_Ds using the equation above. In both our laboratory and others [9, 39], it has been shown that although the absolute RU values change with temperature, analyses of bulk shifts or maximal binding signals following zero adjustment indicate that they do not differ significantly as the temperature changes. This approach was used instead of a more extensive analysis involving the determination of K_D at each temperature by injecting different concentrations of scTCR, as the low affinity of the G99A-pMHC interaction would necessitate the use of large amounts of recombinant protein. In addition, the use of this approach allowed us to carry out experiments on the same sensor chip over the entire temperature range without significant loss of ligand activity.

Loss of ligand activity during the course of the experiments [40] was assessed using repeat injections at the beginning and end of series of analyte injections at different temperatures, and was negligible compared with the change in binding signal as a function of temperature. For most of the temperatures used, duplicate injections of analyte were carried out. The effect of temperature on the TCR-pMHC interaction was also analyzed for two preparations of G99A scTCR with similar results. The K_D values at different temperatures were used to derive the free energy change, ΔG°. To determine ΔH°, ΔS° and ΔC_p°, ΔG° vs. temperature plots were fitted using linear regression as described in [9, 16] to the nonlinear form of the van't Hoff equation:

$$\Delta G_T^\circ = \Delta H_{T_0}^\circ + \Delta C_p^\circ(T - T_0) - T\Delta S_{T_0}^\circ - T\Delta C_p^\circ \ln(T/T_0) \quad [41].$$

In addition, ln K_D vs. 1/T plots were used to calculate the van't Hoff enthalpy of the G99A-pMHC interaction by determining the slope (slope = -ΔH/R) of the linear region of the plot (16–28°C) as described in [17]. This resulted in an estimate for ΔH (-20.7 kcal/mol) that is similar to that determined from fitting to the nonlinear form of the van't Hoff equation (Table 1).

Acknowledgements: We are indebted to Fernando Mateos, James Johnson and Heath McCullough for their excellent assistance with these studies. This work was supported by grants from the NIH (RO1 AI 42949) and National Multiple Sclerosis Society (RG 2411).

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