3. 青山道彦, 吉岡靖雄, 山下浩平, 潘　慧燕, 小椋健正, 平　茉由, 長野一也, 阿部康弘, 鎌田春彦, 角田慎一, 鍋師裕美, 吉川友章, 堤　康央：新規ナノ薬物送達担体の開発を目指した細胞内ナノ動態に関する基礎的検討. 日本薬学会第 132 年会. 札幌（北海道）, 2012 年 3 月.

H. 知的財産権の出願・登録状況

①特許取得

該当事項無し

②実用新案登録

該当事項無し

③その他

該当事項無し

I. 研究協力者

該当事項無し

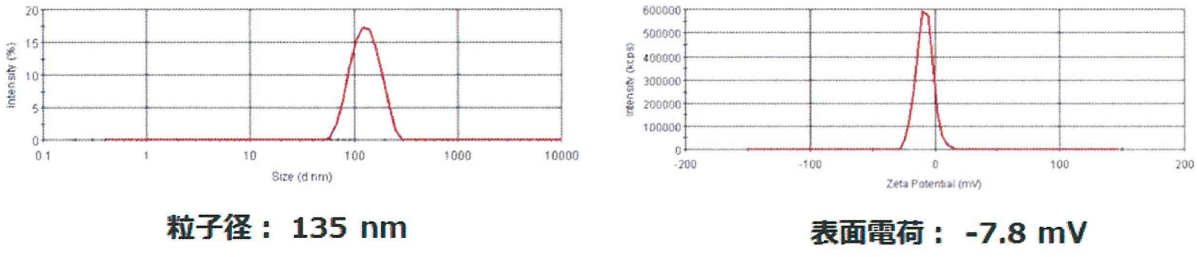

図 1. PVP-C_{60} フラーレンの物性評価. PVP-C_{60} フラーレンの粒子径、表面電荷（ゼータ電位）を評価した。粒子径は動的光散乱法で、ゼータ電位はレーザードップラー法で測定した。

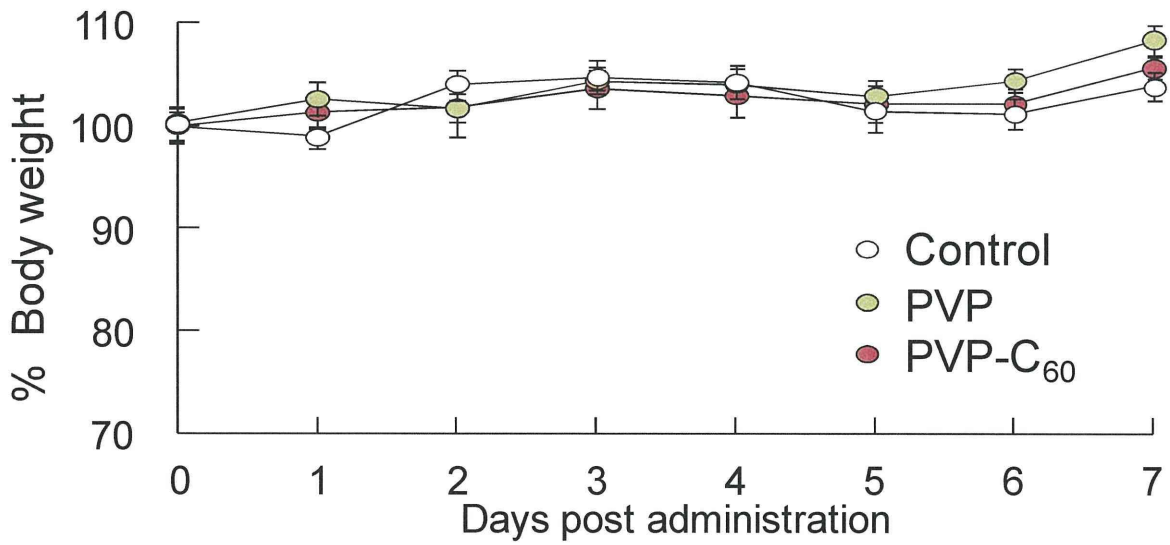

図 2. 体重変化. C57BL/6 マウスに超純水で溶解した PVP-C_{60} フラーレン溶液及び PVP 溶液を 7 日間連続で投与し、投与期間中、毎日体重を測定した。

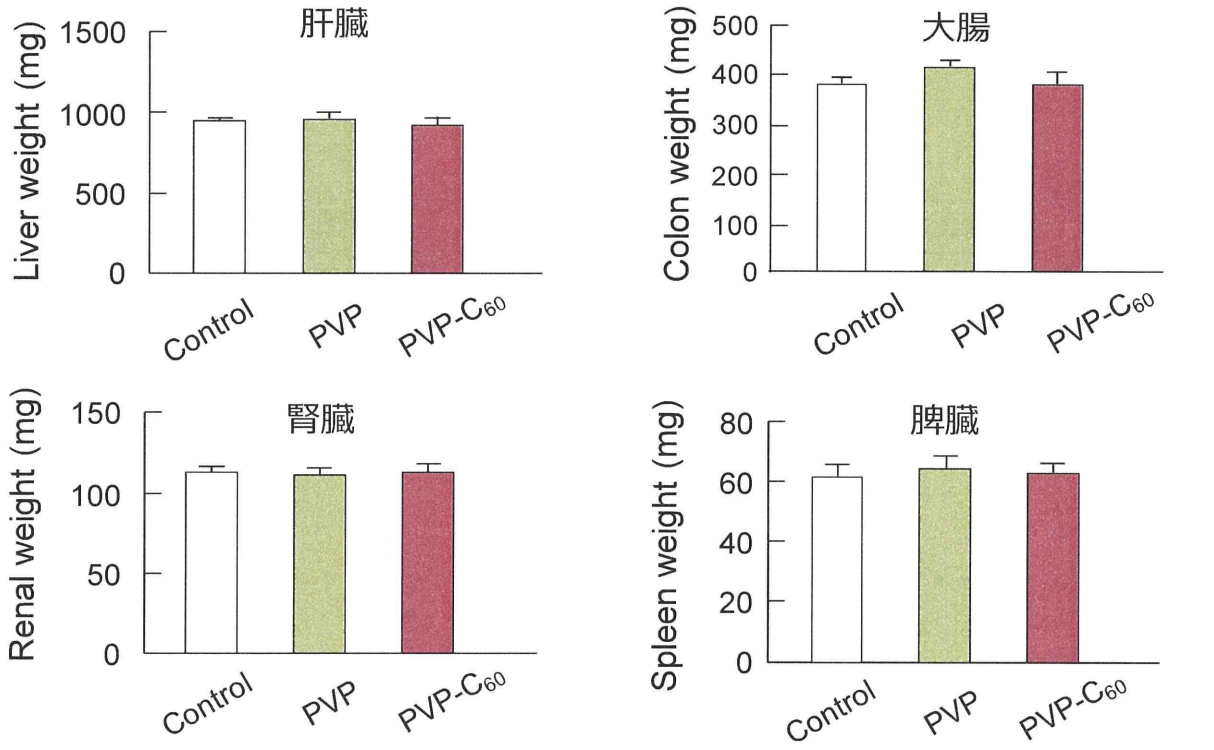

図 3. 臓器重量測定. C57BL/6 マウスに超純水で溶解した PVP-C_{60} フラーレン溶液及び PVP 溶液を 7 日間連続で投与し、投与終了後に臓器重量を測定した。

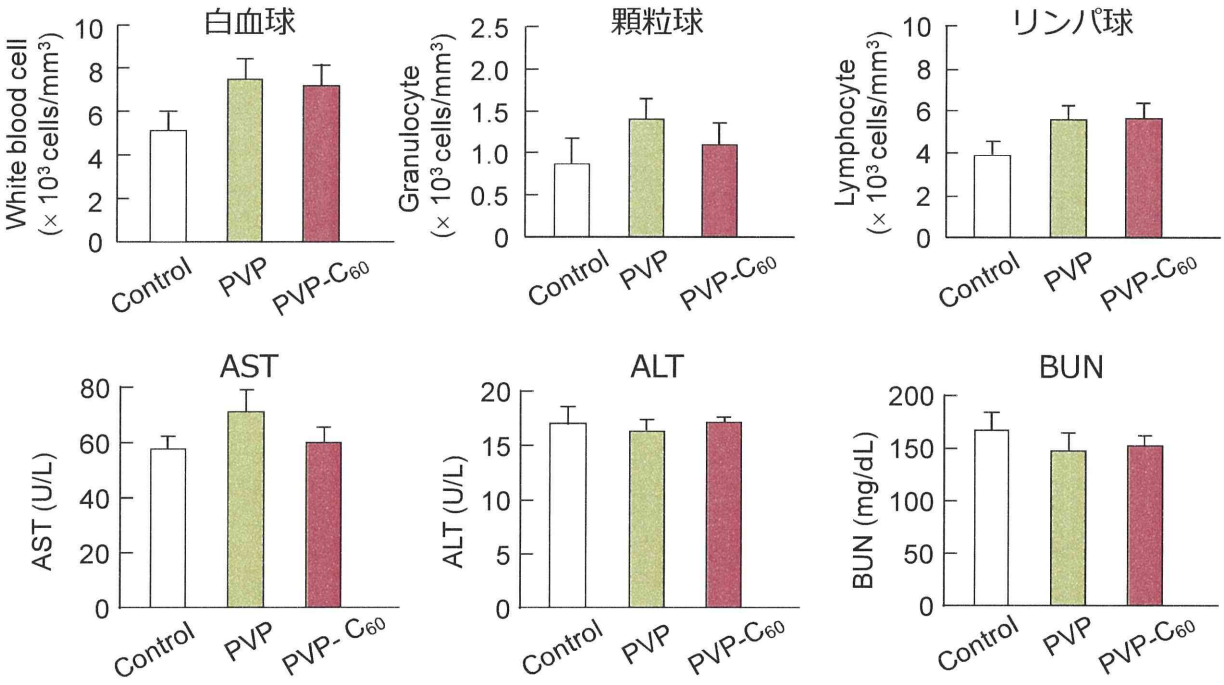

図 4. 生化学的検査. C57BL/6 マウスに超純水で溶解した PVP-C_{60} フラーレン溶液及び PVP 溶液を 7 日間連続で投与し、投与終了後に血球数測定、生化学的検査を実施した。

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Narimatsu S, Yoshioka Y, Morishige T, Yao X, Tsunoda S, Tsutsumi Y, Nishimura MI, Mukai Y, Okada N, Nakagawa S.	Structure–activity relationship of T-cell receptors based on alanine scanning	Biochem Biophys Res Commun	415	558-62	2011

Biochemical and Biophysical Research Communications 415 (2011) 558–562

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Structure–activity relationship of T-cell receptors based on alanine scanning

Shogo Narimatsu [a,1], Yasuo Yoshioka [b,c,*,1], Tomohiro Morishige [a], Xinglei Yao [a,d], Shin-ichi Tsunoda [c,e], Yasuo Tsutsumi [b,c,f], Michael I Nishimura [g], Yohei Mukai [a], Naoki Okada [a], Shinsaku Nakagawa [a,b,*]

[a] Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan
[b] The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[d] Institute of Pharmaceutics, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, China
[e] Laboratory of Biomedical Innovation, Graduate school of Pharmaceutical Sciences, Osaka University, 7-6-8 Saito-asagi, Ibaraki, Osaka 567-0085, Japan
[f] Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan
[g] Division of General Surgery, Department of Surgery, Medical University of South Carolina, Charleston, SC, USA

ARTICLE INFO

Article history:
Received 17 October 2011
Available online 31 October 2011

Keywords:
Alanine-scanning
Melanoma
Protein engineering
T-cell receptor

ABSTRACT

T-cell receptors (TCR) recognize complexes between human leukocyte antigens (HLA) and peptides derived from intracellular proteins. Their therapeutic use for antigen targeting, however, has been hindered by the very low binding affinity of TCRs, typically in the 1- to 100-μM range. Therefore, to construct mutant TCRs with high binding affinity, we need to understand the relationship between the structure and activity of these molecules. Here, we attempted to identify the amino acids of the TCR that are important for binding to the peptide/HLA complex. We used a TCR that recognizes complexes between HLA-A*0201 and the peptide from tyrosinase, antigen overexpressed in melanoma. We changed 16 amino acids in the third complementarity-determining region within the TCR to alanine and examined the effect on binding affinity. Five alanine substitutions decreased the binding affinity to below 10% compared with that of wild-type TCR. In contrast, one alanine substitution caused a faster on-rate and slower off-rate, and increased the binding affinity to three times that of the wild-type TCR. Our results provide fundamental information for constructing mutant TCRs with high binding affinity.

1. Introduction

The T-cell receptor (TCR) is a membrane-bound disulfide-linked heterodimer consisting of an α and a β chain [1–3]. Each chain comprises a constant region and a variable region with four framework regions and three complementarity-determining regions (CDR1 to CDR3) [1–3]. TCRs are expressed on T lymphocytes and recognize small peptide antigens on the surfaces of host cells *via* the major histocompatibility complex [MHC; also called the human leukocyte antigen (HLA) system in humans] [1–3]. These peptides consist of about 10 amino acids derived from intracellularly expressed or exogenous proteins. Accordingly, the use of TCRs for antigen-targeting therapy has been explored. In fact, adoptive immunotherapies that make use of T-cells expressing TCRs against specific intracellular cancer antigens currently represent an area of intense interest in the field of cancer treatment [4–6]. In addition, TCRs conjugated to cytokine may provide novel cytokine therapies, because they also target intracellularly expressed proteins [7–9].

However, TCRs generally exhibit much lower affinities for their peptide-MHC complexes ($K_D = 10^{-4}$–10^{-7} M) than antibodies do for their antigens ($K_D = 10^{-7}$–10^{-12} M) [10,11]. Therefore, to develop TCR-based therapeutic interventions, mutant TCRs with high affinities are needed. One approach to the augmentation of TCR affinity is to selectively alter the amino acids of the CDR1, 2, and 3 loops in the TCRs [12,13]. The amino acids of CDR1 and 2 interface with peptide-MHC complexes, predominantly through the MHC rather than the peptide. In contrast, CDR3 amino acids interact with the peptide as it lies in the MHC groove, leading to the belief that CDR3 is an important region for peptide-MHC complex binding [14,15]. However, which CDR3 amino acids are important for this binding is not yet known.

Here, we used alanine scanning of CDR3 amino acids to determine which amino acids are required for peptide-HLA complex binding. Our results provide fundamental information for constructing mutant TCRs with high binding affinity.

* Corresponding authors at: The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8233. (Y. Yoshioka), Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8179 (S. Nakagawa).
E-mail addresses: yasuo@phs.osaka-u.ac.jp (Y. Yoshioka), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).
[1] Each author contributed equally to the work.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.10.092

2. Materials and methods

2.1. Cloning of TCR chains

We used TCR_{tyr} specific for the complex between HLA-A*0201 and the $tyrosinase_{368-376}$ peptide (YMDGTMSQV) [16–18]. We cloned the extracellular domains of the α and β chain sequences of TCR_{tyr} into pET15b plasmids (Novagen, Darmstadt, Germany) separately. Two-step PCR amplification of the α chain was performed by using three primers: primer 1,5′-gatataccatggcccttgctaagaccac ccagcccatctctatggactcatatgaaggacaagaag-3′; primer 2,5′-ctgatgtgta tatcacagacaaatgcgtgctagacatgaggtctatggac-3′; and primer 3,5′-cc ggatcctggagttattaggaactttctgggctggggaagaagg-3′. By using these three primers, we changed threonine 162 of the TCR_{Tyr} α chain to a cysteine codon and the native inter-chain cysteine codon to a TAA stop codon. In addition, three-step PCR amplification of the β chain was performed by using four primers: primer 4,5′-gatataccatggcc gatgctggaatcacccagagccc-3′; primer 5,5′-acccaaaaggccacactggtgtg cctggccaccggtttcttccctgaccacgtggagctgagctggtgggtgaatgggaaggaggt gcacagtggggtctgcacggacccgcagcccctc-3′; primer 6,5′-ccggatcctcga gttattagtctgctctaccccaggcctcggc-3′; and primer 7,5′-gaccctcaggc ggctgctcagagcgtatctggagtcattgagggcgggctgctccttgaggggctgcgggtccg tgcag-3′. By using these 4 primers, we changed serine 177 of the TCR_{Tyr} β chain to a cysteine codon; we also changed the native inter-chain cysteine codon to a TAA stop codon and cysteine 195 of the TCR_{Tyr} β chain, which is not used for disulfide binding, to an alanine codon.

2.2. Expression and refolding of TCR_{tyr} protein

pET15b plasmids separately encoding the α and β chains were prepared and used to transform *Escherichia coli* BL21(DE3) cells (Stratagene, Cedar Creek, TX) for the expression of recombinant proteins. Expression was induced by adding 1 mM Isopropyl β-D-1-thiogalactpyranoside and incubating at 37 °C for 6 h in Terrific Broth (Invitrogen, Carlsbad, CA) containing 0.4% glucose, 1.68 mM MgSO4, and 100 mg/mL ampicillin; all products accumulated as inclusion bodies. Inclusion bodies prepared from cell lysates were washed in 25% Triton-X 100 and solubilized in 6 M guanidine–HCl, 10 mM dithiothreitol, and 10 mM ethylenediaminetetra-acetate, buffered with 50 mM Tris (pH 8.1). TCR_{tyr} was refolded by rapid dilution of a mixture of the dissolved α and β chain inclusion bodies into 5 M urea, 0.4 M L-arginine, 100 mM Tris (pH 8.1), 3.7 mM cystamine, and 6.6 mM β-mercapoethylamine to a final concentration of 60 mg/L for 36 h at 4 °C.

2.3. Purification of TCR_{tyr} protein

After being dialyzed against demineralized water and 10 mM Tris (pH 8.1) at 4 °C, the refolded proteins were filtered and purified by use of ion-exchange chromatography (Q Sepharose Fast Flow; GE Healthcare, Buckinghamshire, UK). The column was washed with 10 mM Tris (pH 8.1) before elution with a 0- to 500-mM NaCl gradient in the same buffer. The elutes were further purified over a HiLoad Superdex 200PG column (GE Healthcare) equilibrated with PBS (pH 7.4). Fractions comprising the main peak were pooled and analyzed further. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the final purified TCR_{tyr} was conducted under reducing and non-reducing conditions; proteins were stained with Coomassie brilliant blue (CBB).

2.4. Determination of binding affinity by using surface plasmon resonance (SPR) analysis

SA Sensor chips (BIAcore, St Albans, UK) were coated with streptavidin covalently immobilized on a carboxymethylated dextran matrix, and $tyrosinase_{368-376}$/HLA-A*0201 complexes conjugated to biotin (MBL, Nagoya, Japan) were passed over individual flow cells until the response measured about 1000 response units (RU). TCR_{tyr} protein diluted in HBS-EP running buffer (GE Healthcare) was passed over the $tyrosinase_{368-376}$/HLA-A*0201 complexes for 2 min at a flow rate of 20 μL/min. During the dissociation phase, HBS-EP was run over the sensor chip for 10 min at a flow rate of 20 μL/min. The data obtained were evaluated by using BIAevaluation 4.1 software (GE Healthcare) to apply a 1:1 Langmuir binding model. The sensorgrams were fitted globally over the range of injected concentrations and simultaneously over the association and dissociation phases. To evaluate specificity, we also conducted experiments with MART-1 $peptide_{26-35}$ (ELAGIGILTV)/HLA-A*0201 complexes conjugated to biotin (MBL).

2.5. Generation of alanine scanning TCR_{tyr} mutants

To create alanine scanning TCR_{tyr} mutants, we used the α and β chains in the pET15b plasmids as templates with a KOD-plus mutagenesis kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions. The following four amino acid residues of the α chain CDR3 (Lα90, Vα91, Aα92, and Lα93) and 12 amino acid residues of the β chain CDR3 (Aβ93, Iβ94, Sβ95, Pβ96, Tβ97, Eβ98, Eβ99, Gβ100, Gβ101, Lβ102, Iβ103, and Fβ104) were mutated to alanine. The third amino acid of the α chain CDR3 and the first amino acid of the β chain CDR3 were alanine residues initially, so two alanine residues were changed to glycine residues. Together with wild-type TCR_{tyr} ($wtTCR_{tyr}$), the alanine scanning TCR_{tyr} mutants were produced, purified, and confirmed by SDS–PAGE. Their binding kinetics were analyzed by using SPR analysis.

2.6. Statistical analysis

All data are represented as the means ± SD and differences were compared by using Student's t-test.

3. Results and discussion

3.1. Construction of recombinant TCR_{tyr} protein

Several approaches have been used to generate recombinant TCR proteins; however, there is no universally applicable method for the production of a broad range of TCR proteins [19,20]. Boulter et al. developed a generic recombinant TCR protein production method in which disulfide bond-linked TCRs are generated by introducing a cysteine residue within each of the TCR constant regions [21]. Thus, the TCR proteins can refold from inclusion bodies by using *E. coli* to yield large amounts of soluble, stable, and functional TCRs. First, we attempted to generate recombinant TCR_{tyr} proteins by using an *E. coli* system. TCR_{tyr} genes were cloned into the α and β chain constructs that contained engineered cysteines at positions Tα162 and Sβ177, respectively, by a method similar to that of Boulter et al. [21]. In addition, a free cysteine in the constant domain of the β chain was mutated to alanine to facilitate *in vitro* refolding. The α and β chains were expressed by using *E. coli* strain BL21(DE3) cells separately containing the pET15b plasmids that encoded the α and β chains. After the *E. coli* were lysed, the soluble and insoluble fractions were analyzed by using SDS–PAGE, which confirmed that the α and β chains were present only in the insoluble fraction (data not shown). After solubilization and refolding of the inclusion bodies, we purified the TCR_{tyr} by ion exchange and gel filtration chromatography (Fig. 1A). The protein was eluted as a single major peak at an elution time of ~80 min. Examination of the molecular weight markers indicated that the molecular weight of the protein was about 50 kDa. After fractions

corresponding to the main peak were collected and pooled, the purified protein was analyzed by using CBB staining after SDS–PAGE under reducing and non-reducing conditions (Fig. 1B). The α and β chains ran separately under reducing conditions. Under non-reducing conditions, the introduced disulfide bond held the chains together such that they ran as a single band. These results show that the recombinant TCR_{tyr} formed a heterodimer consisting of α and β chains.

3.2. Binding affinity of TCR_{tyr} to tyrosinase$_{368-376}$/HLA-A*0201 complexes

To measure the binding affinity of TCR_{tyr} to tyrosinase$_{368-376}$/HLA-A*0201 complexes, we performed an SPR analysis with a BIAcore 2000. The TCR_{tyr} bound to the tyrosinase$_{368-376}$/HLA-A*0201 complexes (Fig. 1C), but it failed to bind to MART-1_{26-35}/HLA-A*0201 complexes (Fig. 1D). The equilibrium association constant (KD) of TCR_{tyr} to tyrosinase$_{368-376}$/HLA-A*0201 complexes was 2.7 μM (Table 1). This value is almost equivalent to that of TCRs that specifically recognize other peptide-MHC complexes [11]. These results suggest that the recombinant TCR_{tyr} protein has both binding affinity and specificity for tyrosinase$_{368-376}$/HLA-A*0201 complexes.

3.3. Alanine scanning of the 16 amino acids in CDR3

To define the amino acids of CDR3 that are important for deep binding of the tyrosinase$_{368-376}$/HLA-A*0201 complexes, we performed alanine scanning mutagenesis of the TCR_{tyr}. We attempted to create 16 alanine substitutions, which we then purified by ion exchange and gel filtration chromatography (data not shown). We were able to produce all of the desired mutants except for Vα91A. Therefore, we could not assess the role of this mutation. The purified proteins were analyzed by using CBB staining after SDS–PAGE under reducing and non-reducing conditions. The created point mutants formed the same heterodimeric structure that $wtTCR_{tyr}$ formed (data not shown). We confirmed that all substitution mutants could not bind to MART-1_{26-35}/HLA-A*0201 complexes (data not shown). When we examined the interaction of the mutants with tyrosinase$_{368-376}$/HLA-A*0201 complexes (Fig. 2), we found that eight of the substitution mutants, Aα92G, Lα93A, Iβ94A, Pβ96A, Tβ97A, Eβ98A, Iβ103A, and Fβ104A, showed significantly decreased binding responses to the tyrosinase$_{368-376}$/HLA-A*0201 complexes compared with those of $wtTCR_{tyr}$. In particular, Aα92G and Fβ104A did not bind at all. We also found that the KDs of Tβ97A, Eβ98A and Iβ103A decreased to less than 10% relative to $wtTCR_{tyr}$ (Table 1). On the other hand, Sβ95A, Eβ99A, Gβ100A and Lβ102A showed increased binding responses to the tyrosinase$_{368-376}$/HLA-A*0201 complexes compared with those of $wtTCR_{tyr}$. Furthermore, only Gβ100A raised k_{on} and reduced k_{off} compared with those of $wtTCR_{tyr}$, resulting in the highest relative binding affinity (315%). We concluded that Aα92, Tβ97, Eβ98, Gβ100, Iβ103 and Fβ104 were key residues for the interaction of TCR_{tyr} with tyrosinase$_{368-376}$/HLA-A*0201 complexes. In contrast, the relative bindings of the other nine substitutions (Lα90A, Lα93A, Aβ93G, Iβ94A, Sβ95A, Pβ96A, Eβ99A, Gβ101A, and Lβ102A) ranged from 12.8% to 171.7% compared with that in $wtTCR_{tyr}$ (Fig. 2 and Table 1). We concluded that these residues were not important for binding to the tyrosinase$_{368-376}$/HLA-A*0201 complexes.

The detailed crystal structure of the TCR-HLA complex would be useful for understanding binding modes and kinetic behaviors. However, high-resolution crystal structure of the TCR_{tyr}-HLA complex has not yet been solved and the number of different crystal structures of TCR-HLA complexes is very small [22]. In addition, it is difficult to speculate as to the precise interaction mode of a TCR with a given HLA from the crystal structure of other TCR-HLA complexes, because the interaction mode of the TCR, especially CDR3, differs with each HLA [22]. Therefore we could not discuss why only Gβ100A raised k_{on} and reduced k_{off} compared with the k_{on} and k_{off} values of $wtTCR_{tyr}$, resulting in the highest relative binding affinity. Structural information on a large number of TCR-HLA complexes, including that from this study, will help us

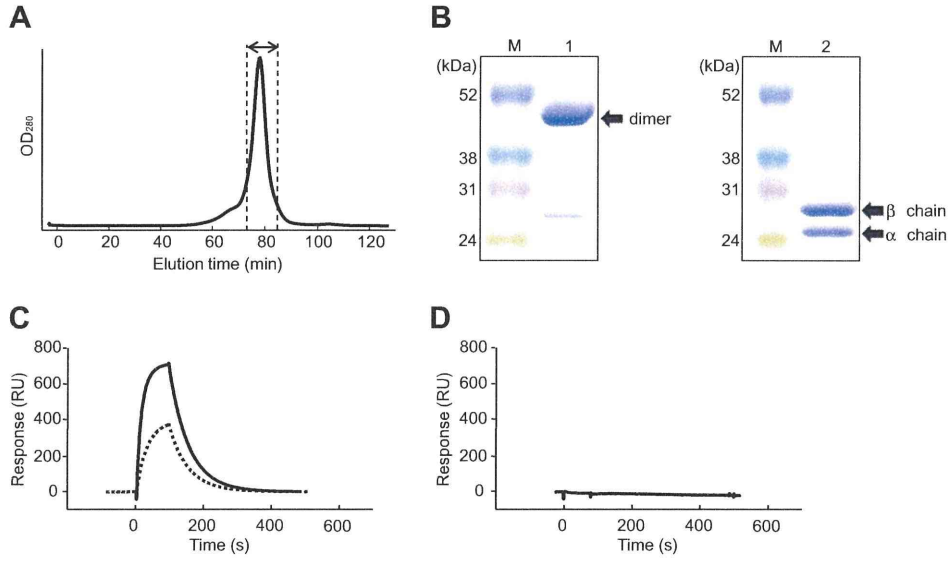

Fig. 1. Construction of $wtTCR_{tyr}$ protein expressed in *E. coli*. (A) Purification of $wtTCR_{tyr}$ by size-exclusion chromatography. Refolded $wtTCR_{tyr}$ was purified by gel filtration chromatography on a Superdex 200PG 16/60 column at a flow rate of 1 mL/min in PBS. (B) SDS–PAGE of pooled fractions. TCR_{tyr} was analyzed by using CBB staining after SDS–PAGE under non-reducing and reducing conditions. Samples were applied to a 4% to 20% SDS–polyacrylamide gel and strained with CBB. Lane M, molecular weight standards; lane 1, $wtTCR_{tyr}$ under non-reducing conditions; lane 2, $wtTCR_{tyr}$ under reducing conditions. Comparison of BIAcore sensorgrams obtained by the binding response to (C) tyrosinase$_{368-376}$/HLA-A*0201 complexes and (D) MART-1_{26-35}/HLA-A*0201 complexes. Duplicate injections of 6.8 μM (—) or 3.4 μM (––) $wtTCR_{tyr}$ were passed over the immobilized peptide/HLA-A*0201 complexes at a flow rate of 20 mL/min. The amount of TCR_{tyr} bound to the peptide/HLA-A*0201 complexes was recorded in response units (RU). The sensorgrams shown were normalized by subtracting the control surface sensorgram.

Table 1
Evaluation of the kinetic parameters of alanine substitution TCR_{tyr} for binding to tyrosinase$_{368-376}$/HLA-A*0201 complexes by using surface plasmon resonance (SPR) analysis. Each kinetic parameter was calculated from the respective sensorgram by using BIA evaluation 4.1 software.

Clone	k_{on} [a] ($\times 10^3$ 1/Ms)	k_{off} [b] ($\times 10^{-3}$ 1/s)	K_D [c] (μM)	Relative binding affinity [d] (%)
$wtTCR_{tyr}$	5.3 ± 0.4	14.2 ± 0.6	2.7 ± 0.1	100
Lα90A	7.3 ± 0.6	22.1 ± 0.8	3.0 ± 0.3	88.7
Aα92G	N.D.	N.D.	N.D.	N.D.
Lα93A	n.d.	n.d.	21.0 ± 1.1**	12.8
Aβ93G	11.1 ± 2.7$^{\#}$	38.6 ± 0.2^{++}	3.6 ± 0.8	75.0
Iβ94A	n.d.	n.d.	17.6 ± 2.0**	15.3
Sβ95A	11.0 ± 2.9$^{\#}$	16.6 ± 0.3^{++}	1.6 ± 0.3**	171.7
Pβ96A	6.8 ± 3.2	76.0 ± 0.3^{++}	12.8 ± 5.3*	21.2
Tβ97A	0.1 ± 0.0$^{\#\#}$	29.2 ± 3.7^{++}	658.1 ± 63.6**	0.4
Eβ98A	n.d.	n.d.	73.2 ± 15.6**	3.7
Eβ99A	9.9 ± 0.2$^{\#\#}$	17.7 ± 0.2^{++}	1.8 ± 0.0**	150.4
Gβ100A	9.6 ± 1.9$^{\#}$	8.1 ± 0.1^{++}	0.9 ± 0.1**	314.5
Gβ101A	5.9 ± 1.2	17.3 ± 1.2^{+}	3.0 ± 0.4	90.1
Lβ102A	9.1 ± 1.6$^{\#}$	14.9 ± 0.3	1.7 ± 0.2**	161.5
Iβ103A	n.d.	n.d.	102.1 ± 24.4**	2.6
Fβ104A	N.D.	N.D.	N.D.	N.D.

All data are represented as the means ± SD.
[a] k_{on} is the association kinetic constant.
[b] k_{off} is the dissociation kinetic constant.
[c] K_D is the equilibrium association constant.
[d] Relative binding affinity values are calculated as 100 × K_D ($wtTCR_{tyr}$)/K_D (mutant). N.D., not detectable; n.d., no data.
[#] $P < 0.05$.
[##] $P < 0.01$ versus value for k_{on} of $wtTCR_{tyr}$.
[+] $P < 0.05$.
[++] $P < 0.01$ versus value for k_{off} of $wtTCR_{tyr}$.
[*] $P < 0.05$.
[**] $P < 0.01$ versus value for K_D of $wtTCR_{tyr}$.

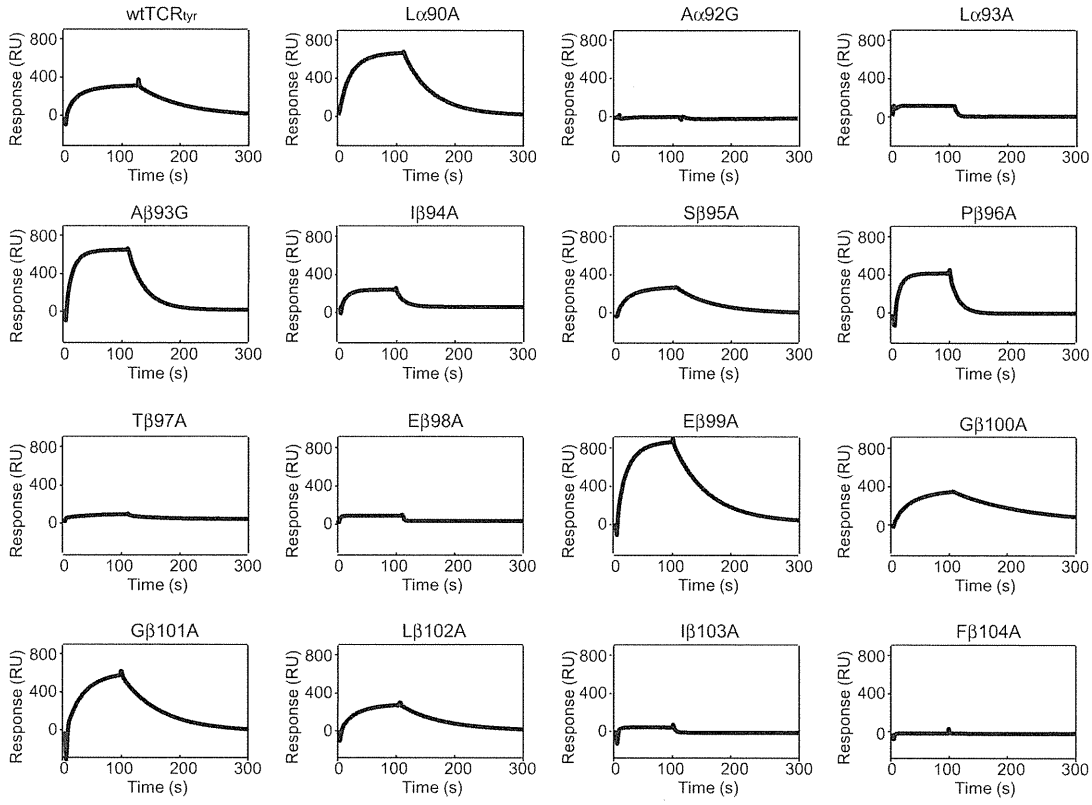

Fig. 2. BIAcore sensorgrams of alanine substitution TCR_{tyr} obtained by the binding response to tyrosinase$_{368-376}$/HLA-A*0201 complexes. Duplicate injections of 3.9 μM TCR_{tyr} were passed over the immobilized peptide/HLA-A*0201 complexes at a flow rate of 20 μL/min. The sensorgrams shown were normalized by subtracting the control surface sensorgram. The amount of protein bound to the surface was recorded in RU.

to understand the precise interaction mode of the TCR with HLA in the future.

Yi Li et al. constructed high-affinity TCRs ($K_D = 2.5 \times 10^{-9}$ M) by using phage display [12]. Their TCRs were generated by extensive mutation of the TCR CDR loops and loop-flanking residues. In contrast, Dunn et al. acquired high-affinity TCRs ($K_D = 1.3 \times 10^{-9}$ M) by mutating only CDR2 amino acids [13]. Although CDR2s contact only the HLA surface and not the bound peptide, the CDR2 mutations did not compromise the selectivity for the bound peptide antigen. We selected CDR3 because its amino acids residues are thought to be most involved in the binding to the peptide-HLA complexes. From here, we plan to similarly characterize the amino acids of CDR1 and 2 to provide the same basic information about them as we were able to obtain about the amino acids of CDR3.

Here, we defined several important amino acids for peptide-HLA complex binding by using alanine scanning of CDR3 amino acids. We showed that the binding affinities of five alanine substitutions dropped to below 10% compared with that of $wtTCR_{tyr}$. Furthermore, one alanine substitution reduced the dissociation kinetic constant compared with that of $wtTCR_{tyr}$ and, as a result, improved the binding affinity. We believe that these results provide basic information of value for a variety of potential biomedical applications of TCRs.

Acknowledgments

The authors declare no conflict of interests. This study was supported in part by grants from the Ministry of Health, Labor, and Welfare of Japan; by a Research on Health Sciences Focusing on Drug Innovation grant from the Japan Health Sciences Foundation; and by the Global COE Program "In Silico Medicine" at Osaka University.

References

[1] R.K. Wilson, E. Lai, P. Concannon, R.K. Barth, L.E. Hood, Structure, organization and polymorphism of murine and human T-cell receptor alpha and beta chain gene families, Immunol. Rev. 101 (1988) 149–172.
[2] J.L. Strominger, Developmental biology of T cell receptors, Science 244 (1989) 943–950.
[3] S.J. Turner, P.C. Doherty, J. McCluskey, J. Rossjohn, Structural determinants of T-cell receptor bias in immunity, Nat. Rev. Immunol. 6 (2006) 883–894.
[4] H.W. Kessels, M.C. Wolkers, M.D. van den Boom, M.A. van der Valk, T.N. Schumacher, Immunotherapy through TCR gene transfer, Nat. Immunol. 2 (2001) 957–961.
[5] C. Rossig, C.M. Bollard, J.G. Nuchtern, C.M. Rooney, M.K. Brenner, Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy, Blood 99 (2002) 2009–2016.
[6] V. Karanikas, C. Lurquin, D. Colau, N. van Baren, C. De Smet, B. Lethe, T. Connerotte, V. Corbiere, M.A. Demoitie, D. Lienard, B. Dreno, T. Velu, T. Boon, P.G. Coulie, Monoclonal anti-MAGE-3 CTL responses in melanoma patients displaying tumor regression after vaccination with a recombinant canarypox virus, J. Immunol. 171 (2003) 4898–4904.
[7] K.F. Card, S.A. Price-Schiavi, B. Liu, E. Thomson, E. Nieves, H. Belmont, J. Builes, J.A. Jiao, J. Hernandez, J. Weidanz, L. Sherman, J.L. Francis, A. Amirkhosravi, H.C. Wong, A soluble single-chain T-cell receptor IL-2 fusion protein retains MHC-restricted peptide specificity and IL-2 bioactivity, Cancer Immunol. Immunother. 53 (2004) 345–357.
[8] H.J. Belmont, S. Price-Schiavi, B. Liu, K.F. Card, H.I. Lee, K.P. Han, J. Wen, S. Tang, X. Zhu, J. Merrill, P.A. Chavillaz, J.L. Wong, P.R. Rhode, H.C. Wong, Potent antitumor activity of a tumor-specific soluble TCR/IL-2 fusion protein, Clin. Immunol. 121 (2006) 29–39.
[9] J. Wen, X. Zhu, B. Liu, L. You, L. Kong, H.I. Lee, K.P. Han, J.L. Wong, P.R. Rhode, H.C. Wong, Targeting activity of a TCR/IL-2 fusion protein against established tumors, Cancer Immunol. Immunother. 57 (2008) 1781–1794.
[10] S.S. Khandekar, P.P. Brauer, J.W. Naylor, H.C. Chang, P. Kern, J.R. Newcomb, K.P. Leclair, H.S. Stump, B.M. Bettencourt, E. Kawasaki, J. Banerji, A.T. Profy, B. Jones, Affinity and kinetics of the interactions between an alphabeta T-cell receptor and its superantigen and class II-MHC/peptide ligands, Mol. Immunol. 34 (1997) 493–503.
[11] D.K. Cole, N.J. Pumphrey, J.M. Boulter, M. Sami, J.I. Bell, E. Gostick, D.A. Price, G.F. Gao, A.K. Sewell, B.K. Jakobsen, Human TCR-binding affinity is governed by MHC class restriction, J. Immunol. 178 (2007) 5727–5734.
[12] Y. Li, R. Moysey, P.E. Molloy, A.L. Vuidepot, T. Mahon, E. Baston, S. Dunn, N. Liddy, J. Jacob, B.K. Jakobsen, J.M. Boulter, Directed evolution of human T-cell receptors with picomolar affinities by phage display, Nat. Biotechnol. 23 (2005) 349–354.
[13] S.M. Dunn, P.J. Rizkallah, E. Baston, T. Mahon, B. Cameron, R. Moysey, F. Gao, M. Sami, J. Boulter, Y. Li, B.K. Jakobsen, Directed evolution of human T cell receptor CDR2 residues by phage display dramatically enhances affinity for cognate peptide-MHC without increasing apparent cross-reactivity, Protein Sci. 15 (2006) 710–721.
[14] J.B. Reiser, C. Darnault, C. Gregoire, T. Mosser, G. Mazza, A. Kearney, P.A. van der Merwe, J.C. Fontecilla-Camps, D. Housset, B. Malissen, CDR3 loop flexibility contributes to the degeneracy of TCR recognition, Nat. Immunol. 4 (2003) 241–247.
[15] J. Zhou, R. Ma, R. Luo, Y. Sun, X. He, W. Sun, W. Tang, X. Yao, Primary exploration of CDR3 spectratyping and molecular features of TCR beta chain in the peripheral blood and tissue of patients with colorectal carcinoma, Cancer Epidemiol 34, 733–740.
[16] M.I. Nishimura, D. Avichezer, M.C. Custer, C.S. Lee, C. Chen, M.R. Parkhurst, R.A. Diamond, P.F. Robbins, D.J. Schwartzentruber, S.A. Rosenberg, MHC class I-restricted recognition of a melanoma antigen by a human CD4+ tumor infiltrating lymphocyte, Cancer Res. 59 (1999) 6230–6238.
[17] M.I. Nishimura, J.J. Roszkowski, T.V. Moore, N. Brasic, M.D. McKee, T.M. Clay, Antigen recognition and T-cell biology, Cancer Treat. Res. 123 (2005) 37–59.
[18] R.C. Koya, S. Mok, B. Comin-Anduix, T. Chodon, C.G. Radu, M.I. Nishimura, O.N. Witte, A. Ribas, Kinetic phases of distribution and tumor targeting by T cell receptor engineered lymphocytes inducing robust antitumor responses, Proc. Natl. Acad. Sci. USA 107 14286–14291.
[19] B.E. Willcox, G.F. Gao, J.R. Wyer, J.E. Ladbury, J.I. Bell, B.K. Jakobsen, P.A. van der Merwe, TCR binding to peptide-MHC stabilizes a flexible recognition interface, Immunity 10 (1999) 357–365.
[20] B.E. Willcox, G.F. Gao, J.R. Wyer, C.A. O'Callaghan, J.M. Boulter, E.Y. Jones, P.A. van der Merwe, J.I. Bell, B.K. Jakobsen, Production of soluble alphabeta T-cell receptor heterodimers suitable for biophysical analysis of ligand binding, Protein Sci. 8 (1999) 2418–2423.
[21] J.M. Boulter, M. Glick, P.T. Todorov, E. Baston, M. Sami, P. Rizkallah, B.K. Jakobsen, Stable, soluble T-cell receptor molecules for crystallization and therapeutics, Protein Eng. 16 (2003) 707–711.
[22] L. Varani, A.J. Bankovich, C.W. Liu, L.A. Colf, L.L. Jones, D.M. Kranz, J.D. Puglisi, K.C. Garcia, Solution mapping of T cell receptor docking footprints on peptide-MHC, Proc. Natl. Acad. Sci. USA 104 (2007) 13080–13085.