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Humanization of a phosphothreonine peptide-specific chicken antibody by combinatorial library optimization of the phosphoepitope-binding motif

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ABSTRACT

Detection of protein phosphorylation at a specific residue has been achieved by using antibodies, which have usually been raised by animal immunization. However, there have been no reports of the humanization of phosphospecific non-human antibodies. Here, we report the humanization of a chicken pT231 antibody specific to a tau protein-derived peptide carrying the phosphorylated threonine at residue 231 (pT231 peptide) as a model for better understanding the phosphoepitope recognition mechanism. In the chicken antibody, the phosphate group of the pT231-peptide antigen is exclusively recognized by complementarity determining region 2 of the heavy chain variable domain (VH-CDR2). Simple grafting of six CDRs of the chicken antibody into a homologous human framework (FR) template resulted in the complete loss of pT231-peptide binding. Using a yeast surface-displayed combinatorial library with permutations of 11 FR residues potentially affecting CDR loop conformations, we identified 5 critical FR residues. The back mutation of these residues to the corresponding chicken residues completely recovered the pT231-peptide binding affinity and specificity of the humanized antibody. Importantly, the back mutation of the FR 76 residue of VH (H76) (Asn to Ser) was critical in preserving the pT231-binding motif conformation via allosteric regulation of ArgH71, which closely interacts with ThrH52 and SerH52a residues on VH-CDR2 to induce the unique phosphate-binding bowl-like conformation. Our humanization approach of CDR grafting plus permutations of FR residues by combinatorial library screening can be applied to other animal antibodies containing unique binding motifs on CDRs specific to posttranslationally modified epitopes.

1. Introduction

Protein phosphorylation, one of the most common post-translational modifications, is an important cellular event that is involved in the regulation of protein activity by modulation of protein–protein interactions, subcellular localization, and/or stability [1]. In eukaryotes, phosphoryl groups can be attached to the hydroxyl side chain of Ser, Thr, and/or Tyr residues by various kinases [2]. Aberrant phosphorylation is associated with many human diseases, including cancers [3]. Therefore, precise monitoring of the phosphorylation state of proteins at a specific residue is essential to understand its function and roles in the normal and pathological states [2]. Currently, the best phosphorylation state-specific detection agent is antibodies, which have usually been raised by immunization of animals, such as a mouse, rabbit, or chicken, with a synthetic phosphopeptide corresponding to the protein phosphorylated at the targeted site [4].

However, the molecular mechanisms how the phosphospecific antibodies recognize the phosphorylated epitopes are poorly understood. Recently, a chicken pT231 antibody, specifically recognizing a peptide carrying the phosphorylated Thr (pThr) at residue 231 (pT231 peptide) with an amino acid sequence of ^{224}KKVAVVR(pT^{231})PPKSPSSAK^{240} that is associated with the Alzheimer's disease-associated tau protein, was selected from a phage-displayed antibody library constructed from phosphopeptide-immunized chickens [5]. The X-ray crystal complex structure of the chicken antibody with its antigen pT231 peptide revealed a unique phosphoepitope recognition mechanism [5]. The pThr residue of the pT231-peptide was exclusively recognized by the complementarity determining region 2 of the heavy chain variable

Abbreviations: CDR, complementarity determining region; VH, heavy chain variable domain; VL, light chain variable domain.

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domain (VH-CDR2), which has a "bowl-like" nest conformation to tightly and specifically interact with the phosphate group [5]. Intriguingly, the recently reported phosphospecific antibody scaffold for pSer- and pThr-peptide also showed a strikingly similar recognition motif on VH-CDR2 [6].

Antibody humanization refers to grafting antigen-recognizing paratopes of non-human antibodies into a human antibody template while maintaining the parent antigen-binding specificity and affinity. A simple humanization approach is to graft six CDRs of non-human antibodies into the corresponding regions of a human framework (FR) with the sequence most similar to that of the non-human antibody. However, in many cases this approach results in the loss of the original antigen binding affinity because certain FR residues of the donor non-human antibody that affect the conformation of the CDR loops and thereby antigen affinity are not retained in humanized antibodies [7–10]. Therefore, some FR residues that support the proper conformation of CDR loops of the non-human antibody, such as "Vernier zone residues" [11] and "packing residues" [12,13], should be properly grafted together with CDRs to restore antibody binding affinity and specificity. However, exploring one by one more than 20 such FR residues of the VH and VL domains by back mutations with the corresponding donor amino acid residues is laborious and time consuming. Accordingly, the combinatorial FR library and FR shuffling methods, introducing all possible combinations of human and non-human FR residues, have been developed to identify many critical FR residues at once for the preservation of grafted CDR loop conformations [12,14,15].

To date, however, antibody humanization has been reported only for non-human antibodies with intact protein and peptide as antigens. There have been no reports of humanization of antibodies recognizing posttranslationally modified proteins/peptides, including phosphorylated peptides/proteins. Here, we describe the humanization of a pThr-peptide specific antibody, using the chicken pT231 antibody as a model, to better understand the phosphoepitope recognition mechanism. Using a yeast surface-displayed combinatorial library for 11 FR residues, we identified 5 critical FR residues that restored the donor CDR conformations, including the VH-CDR2 containing the pThr-peptide binding motif. The resultant humanized antibodies in an IgG format showed binding affinity and specificity for the pThr-peptide that were comparable to those of the chicken antibody.

2. Materials and methods

2.1. Materials

The C-terminal biotinylated pT231 peptide (^{224}KKVAVVR(pT^{231})PPKSPSSAK^{240}-biotin) derived from the human tau protein [5], the non-phosphorylated T231-peptide (^{224}KKVAVVR(T^{231})PPKSPSSAK^{240}), and off-target pT52-peptide (^{44}SEKQLSQA(pT^{52})AAATNHTTDNGK^{64}-biotin) derived from human lysyl-tRNA synthetase [16] were synthesized with 95% purity (Peptron, Korea). DNAs encoding the initial humanized sequences of VH and VL of the huPT0 clone (Fig. 1) were prepared by DNA synthesis (Bioneer, Korea) and then subcloned in frame into the upstream of the CH1 gene of the pYDS-H and CL gene of the pYDS-L vector (Fig. 2A) [17]. Yeast strains and media composition have been previously described in detail [17]. All of the reagents were of analytic grade.

2.2. Construction of VH and VL gene libraries with FR substitutions on yeast haploid cells

VH and VL gene libraries were constructed by serial overlapping polymerase chain reactions (PCR) with degenerative primers

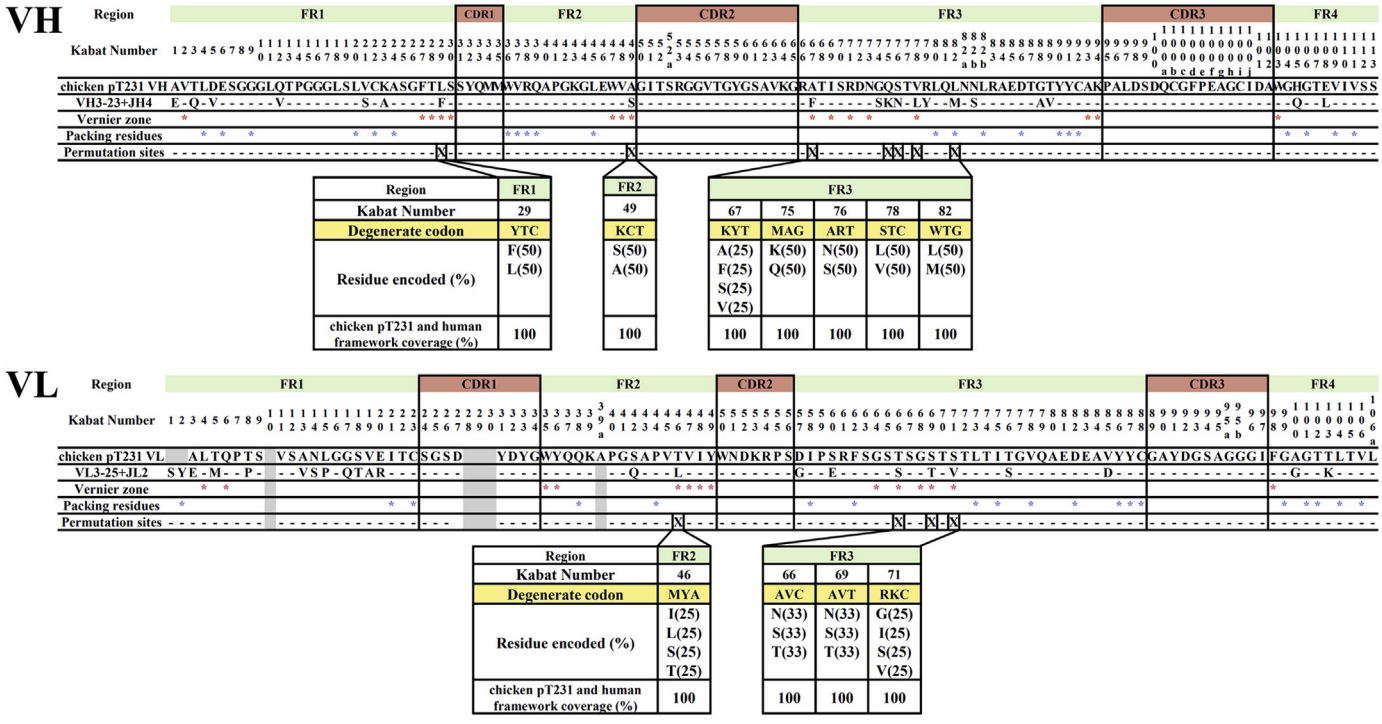

Fig. 1. Amino acid sequence alignment between the VH and VL domains of the chicken pT231 antibody and chosen human germline template, and the library construction strategy involving permutations of the indicated FR residues. The germline VH3-23 and VL3-25 showing the closest sequence homology to the FR sequences of the pT231-peptide-specific chicken pT231 antibody were used as a template for the humanization strategy. The amino acid residues identical between chicken and human FRs are indicated by a hyphen(-). The "Vernier zone residues" and "packing residues" described in the text are marked by asterisks (*). The targeted FR residues, indicated by an X, were permutated with the indicated degenerate codons to cover simultaneously the chicken and human residues at these positions.

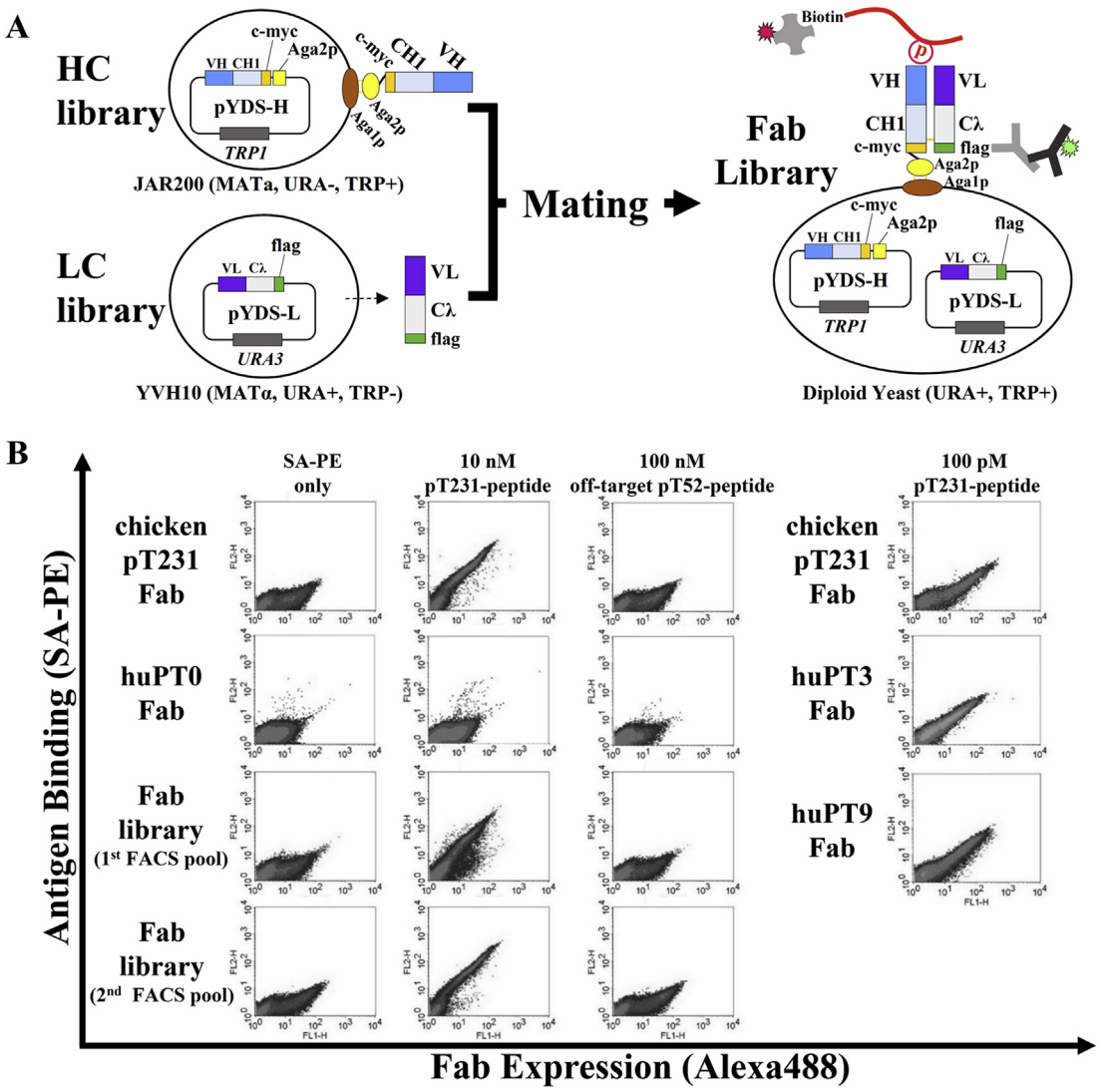

Fig. 2. Construction and screening of yeast diploid cells carrying a combinatorial Fab library with FR permutations. (A) Schematic diagram showing the process of combinatorial Fab library generation on yeast cell surfaces via yeast mating between two haploid cells carrying HC and LC libraries. The Fab assembly was achieved by random pairing of the secreting LC library to the cell wall-anchored HC library by disulfide linkage between Cys residues at the C-terminus of CL and CH1. The Fab assembly of LC and HC was detected by labeling the C-terminal Flag tag of LC and analysis by flow cytometry. (B) Flow cytometric analysis of the yeast surface-displayed Fab antibodies of the chicken pT231 antibody, the initial humanized version of huPT0, FACS-sorted pools, and the two isolated clones (huPT3 and huPT9) for the expression level and antigen binding affinity to the indicated peptides.

designed to introduce mutations at the selected FR residues following methods described previously [17,18]. The list of primers used in this study is in Supplementary Table S1. The amplified VH gene library (12 μg) and linearized pYDS-H vector (4 μg) with *Nhe*I/*Apa*I digestion were co-transformed into the yeast strain *Saccharomyces cerevisiae* JAR200 (MAT**a**) twice [17,19]. Likewise, the VL gene library (12 μg) and linearized pYDS-L vector (4 μg) with *Nhe*I/*Hind*III digestion were co-transformed two times into the *S. cerevisiae* YVH10 (MATα) strain [17]. The transformants were selected directly in single-selective liquid media as described previously [17].

2.3. Combinatorial Fab library construction on diploid cells by yeast mating

Two haploid cells carrying the HC (VH library-CH1) and LC (VL library-CL) library were mated for the generation of the combinatorial Fab library on diploid cells following the optimized yeast mating protocol [17]. Briefly, mating of the two grown haploid cells was performed by mixing equal numbers (~1.5×10^7) of cells in 50 μl of YPD media by vortexing, and then the mixed cells were spread onto a 1-cm^2 area of a pre-warmed YPD agar plate. After incubation for 5 h at 30 °C, yeast cells were scrapped, washed, and then grown on the selective media [17]. The yeast mating efficiency and colony diversity of diploid yeast were calculated as described previously [17].

2.4. Screening of the Fab library

For library screening against the target peptide, two rounds of fluorescence-activated cell sorting (FACS) were carried out with FACS Aria II (Becton & Dickinson, USA) using the biotinylated pT231 peptide following a published protocol [18]. During FACS, the cell surface expression and target peptide binding levels of the Fab library were monitored by indirect double immunofluorescence labeling of the LC C-terminal Flag tag (anti-Flag mouse mAb (Sigma, F-3165)/Alexa Fluor® 488-labeled goat anti-mouse mAb (Invitrogen, A-11001)) and the pT231 peptide (biotinylated substrate/SA-PE) [17,18] (Fig. 2A). The sorting stringency was increased by decreasing the biotinylated pT231 peptide: round 1 = 10 nM and

round 2 = 1 nM. The final, sorted yeast cells were plated on the selective medium, and 30 individual clones were randomly chosen and analyzed to yield the two unique clones. The Fab DNA sequence was identified by yeast colony PCR [17].

2.5. Expression and purification of the humanized IgG antibody

The two isolated Fab clones (huPT3 and huPT9) were reformatted into IgG1 through sub-cloning the HC (VH–CH1) gene into the modified pcDNA 3.4 vector (Invitrogen) carrying the human IgG1 Fc (hinge-CH_2-CH_3) gene and LC (VL-CL) gene into the pcDNA 3.4 vector, as described previously [16,20]. Antibodies were produced by transient transfection of the plasmids into HEK293F cells using the Freestyle 293-F expression and media system (Invitrogen), as described in previous studies [16,20]. Antibodies were purified from the culture supernatants using a CaptivA™ PriMAB protein A column (Repligen, USA) following the manufacturer's protocol.

2.6. Binding analysis by ELISA

Antigen binding affinity and specificity of huPT3 and huPT9 IgG antibodies to the pT231 peptide and the control peptides were determined by ELISA, as described before [18]. Briefly, ELISA plates (Corning, 3390) were coated with 1 μg of peptides (pT231 peptide, non-phosphorylated T231-peptide, or off-target pT52-peptide) for 2 h at 30 °C. Serially diluted humanized IgG antibodies were applied to wells for 1 h at 25 °C. After washing, bound humanized antibodies were detected with anti-human Fc mAb conjugated with HRP (Sigma, A0170) and incubated with TMB liquid substrate (Sigma, T0440) at 25 °C. Absorbance was read at 450 nm on a microplate reader (BioTeK, USA). The equilibrium dissociation binding constants (K_D) were estimated by fitting the raw data into a four-parameter sigmoidal curve on Sigma plot software [18].

2.7. Structure modeling

The Fv (VH:VL) of huPT9 was obtained in GalaxyWeb [21]. A total of seven structures of human Fv (PDB ID: 3IU4, 3U6R, 1KXV, 2VYR, 2EAK, 3QYC, 3R0M) were used as templates to generate the homology model.

3. Results and discussion

3.1. Design of the humanized phosphospecific chicken antibody

The X-ray crystal structure (PDB ID: 4GLR) of the chicken pT231 Fab antibody in complex with the pT231-peptide (^{225}KVAVVR(pT^{231})) has revealed that the phosphoepitope of the pThr231 residue is exclusively recognized by a "bowl-like" binding motif on VH-CDR2 and the rest of the non-phosphorylated sequence (^{225}KVAVVR^{230}) is recognized by the other VH- and VL-CDRs, with VH-CDR3 being the dominant region involved [5]. The binding motif of the phosphorylation site (pThr231), specifically, is composed of five residues, ThrH52, SerH52a, ArgH53, GlyH54, and GlyH55 (where H = HC with Kabat numbering [22]), that form a positively charged pocket to accommodate the phosphate on VH-CDR2 [5]. Accordingly, the conservation of the exquisite conformation of pThr-binding motif on VH-CDR2 is believed to be a key point for successful humanization.

To choose human germline sequences with maximal homology in the FR regions with the chicken antibody, we performed a BLAST search of the amino acid sequences of VH and VL of the chicken pT231 antibody against the human germline NCBI IgBlast database [23]. The chosen sequences of the human germline VH3-23 (DP-47) and JH4 genes for the VH template and the VL3-25 (IGGLL150) and JL2 genes for the VL template showed identities of approximately 68.2% and 64.7% with the VH and VL FR sequences of the chicken antibody, respectively (Fig. 1). Then, the six CDRs of the chicken pT231 antibody were grafted into the corresponding regions of the chosen human FR template, generating an initial humanized clone of huPT0. When huPT0 was expressed in an Fab format on the yeast cell surface, however, it failed to bind to the pT231 peptide (Fig. 2B), indicating that the simple transfer of six CDRs into the homologous human FR scaffold is not enough to recapitulate the CDR conformations of the phosphospecific pT231 antibody.

Certain residues located in the FR residues often critically affect CDR loop conformations, requiring additional donor residual substitutions into the human FR template to maintain antigen binding specificity and affinity in the humanized antibody [7–10]. In particular, some residues in the FR structure closely underlying the CDRs, so called "Vernier zone residues" [11], are known to affect CDR loop conformation. Furthermore, other FR residues partially exposed or buried in the interior β-sandwich structure often contribute to the relative disposition and orientation of the CDRs. These are defined as "packing residues" [12,13] rather than Vernier zone residues. Primary and tertiary structural analyses identified the above mentioned FR residues, including the H29, H49, H67, H78, H82 residues in VH and the L46, L69, and L71 residues (where L = LC with Kabat numbering [22]) in VL that were different between the chicken and human templates, as the target sites for substitutions (Fig. 1). Further, detailed structural analysis of the pT231 antibody complexed with the pT231-peptide antigen indicated that the residues H71, H73, H75, and H76 located in the loop adjacent to VH-CDR2 are critical for the maintenance of the pThr-binding motif. While ArgH71 and AsnH73 were identical, the H75 and H76 residues were different between the chicken and huPT0 antibody. Thus, we added the two H75 and H76 residues as permutation sites in an attempt to maintain the pThr-binding motif, choosing a total of 11 FR residues for permutation sites (Fig. 1).

3.2. Construction and screening of a combinatorial Fab library

Identifying the optimal single or combined back mutation residues for the chosen 11 FR residues using site-directed mutagenesis might be a time-consuming and laborious process. Thus, we sought to construct a yeast surface-displayed combinatorial library with permutations of the chosen 11 residues for the FR fine-tuning of antigen binding affinity and specificity. To introduce all possible combinational substitutions, the targeted 11 FR residues were diversified with the respective degenerate codon to cover both human and chicken amino acids at the residues (Fig. 1 and Table 1).

As illustrated in Fig. 2A, we first constructed HC and LC libraries independently in two haploid cells of opposite mating types, and then the two haploid libraries were mated to generate the combinatorial Fab library [17]. The library in Fab format, consisting of VH-CH1 and VL-Cλ, was constructed to reduce any reformatting problems, referring to the occasional loss of antigen binding affinity and specificity during reformatting of an antibody fragment, such as scFv, into the intact full-length IgG format [24]. The diversity represented by the Fab library was ~4.5×10^7, sufficiently covering the designed, theoretical sequence space of ~3.7×10^4.

To isolate antibodies with high affinity binding to the target antigen, FACS screening was performed using the biotinylated antigen of the pT231 peptide against the yeast surface-displayed Fab library (Fig. 2B) [17,20]. After two rounds of sorting, 30 clones were randomly analyzed to yield two unique high-affinity binders, designated as huPT3 and huPT9 (Fig. 2B). The two clones showed slightly different amino acid substitutions but qualitatively were very similar at the targeted VH FR residues, while sharing a VL with the identical FR mutations (Table 1). Noticeably, the two clones

Table 1
Summary of sequences at the mutated FR residues in humanized huPT3 and huPT9 clones.

Domain	Positions	Chicken pT231 residue	Human residue	Amino acid prevalence of human germline family (%)[a]				huPT3	huPT9
VH	29	L	F	F (85)	V (11)	C (3)	S (1)	F	F
	49	A	S	S (52)	A (27)	G (21)		S	A
	67	A	F	F (96)	L (4)			S	A
	75	Q	K	K (95)	R (3)	N (2)	T (1)	K	K
	76	S	N	N (95)	S (5)			S	S
	78	V	L	L (91)	A (6)	P (2)	M (1)	L	L
	82	L	M	M (96)	K (2)	T (1)		L	M
VL	46	T	L	L (100)				T	T
	66	T	S	S (44)	N (44)	K (6)	T (6)	T	T
	69	S	T	N (61)	T (39)			T	T
	71	S	V	A (56)	V (28)	T (17)		S	S

[a] Prevalence (%) in human VH3 and Vλ3 germline gene family was analyzed in the IMGT database and represented as a percentage. All residues are numbered according to the Kabat scheme [22] and the degenerate codons are compressed using IUPAC notation.

commonly possessed five back-mutated FR residues at H67, H76, L46, L66, and L71 to those of the parent chicken antibody, suggesting that the donor amino acids at the five FR residues are critical to maintaining the CDR conformations. The four residues H67, L46, L66, and L71 are usually classified as Vernier zone residues supporting the proximal CDR conformation, but the H76 residue is not. Since the H76 residue is located in the loop around VH-CDR2, the back mutation seemed to indirectly play a critical role in recovering the pThr-binding motif on VH-CDR2.

3.3. Expression of humanized antibodies in IgG format and determination of their antigen-binding activity

The huPT3 and huPT9 humanized Fab antibodies were converted into the conventional human IgG1 (IgG1 with VH3- Vλ3) by subcloning the VH and VL sequences into the human IgG1 and CL constant domains, respectively [16]. The plasmids encoding HC and LC, respectively, were transiently co-transfected into HEK293F cells to express the IgG antibody [16,20]. Both huPT3 and huPT9 IgG antibodies were well-expressed in the correctly assembled form (Fig. 3A). In the ELISA, the two humanized IgG antibodies bound to only the cognate pT231-peptide antigen, but not to the non-phosphorylated T231-peptide and off-target pT52-peptide (Fig. 3B–D). The K_D values of the two clones for the cognate pT231 peptide were approximately 1.8 nM, comparable to that ($K_D \approx 1.1$ nM) of the parent chimeric human pT231 IgG1 antibody [5]. Taken together, these data suggest that the two humanized IgG antibodies recovered the binding specificity and affinity for the cognate pT231-peptide antigen, indicating that the two clones were successfully humanized with optimal FR retentions of the donor antibody that maintained the pThr-binding motif in VH-CDR2 and the other CDR conformations.

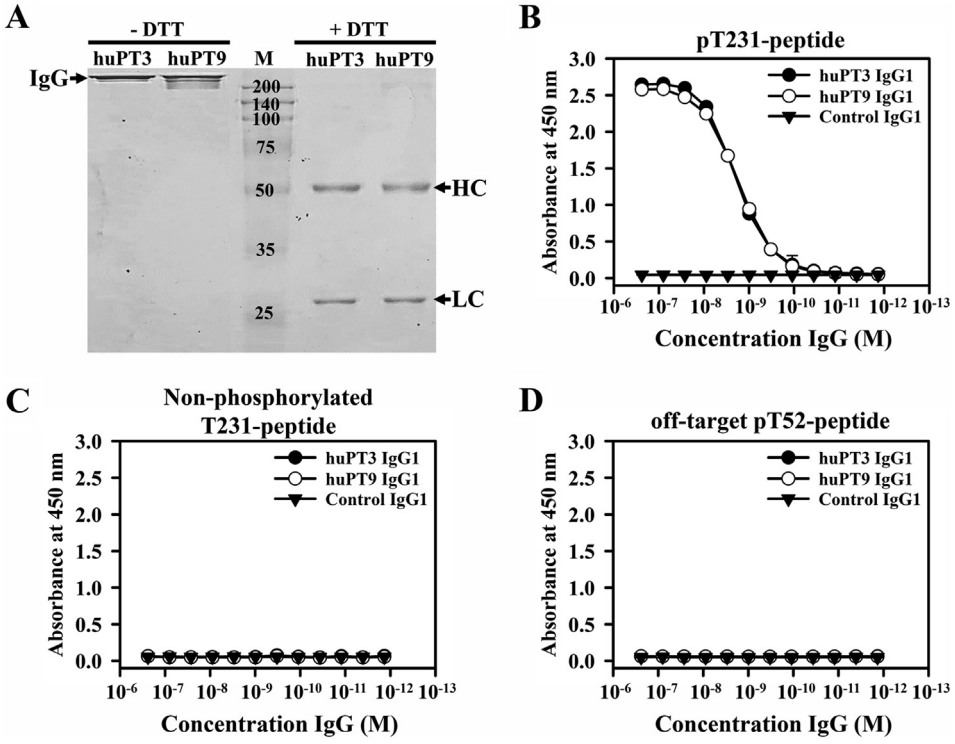

Fig. 3. Binding activity of humanized IgGs to the pT231 peptide. (A) The purified humanized IgG antibodies were separated on a 12% SDS-PAGE gel under non-reducing (−DTT) and reducing (+DTT) conditions and stained with Coomassie brilliant blue stain (R-250). (B–D) ELISA to determine the binding specificity of the humanized huPT3 and huPT9IgG antibodies to the plate-coated pT231-peptide antigen (B), non-phosphorylated T231-peptide (C), and off-target pT52-peptide (D). The anti-Her2 mAb trastuzumab (Herceptin) [20] was employed as a control IgG1 antibody.

3.4. Structural analysis of the pThr-binding motif on VH-CDR2 of the humanized antibody

The complex structure showed that the critical phosphorylation site (pT231) of the pT231-peptide antigen is exclusively recognized by VH-CDR2 of the chicken pT231 antibody [5]. To further understand how the FR back mutations recovered the unique pThr-binding motif on VH-CDR2, we predicted the Fv (VH:VL) structures of huPT9 and also huPT0 for comparison via a template-based-modeling algorithm, while excluding already known chicken antibody structures, including the pT231 antibody, to avoid any bias toward the chicken antibody structure [21]. Interestingly enough, huPT9, but not the initial humanized version of huPT0, exhibited the unique bowl-like conformation on VH-CDR2, very closely superimposing with that of the parent chicken pT231 VH [5] (Fig. 4A). The critical structural difference between huPT0 and huPT9 was the direction of the side chain of the ArgH71 residue. In huPT9, the side chain of ArgH71 is well positioned to form hydrogen bonds with ThrH52 and SerH52a and compose the pT231-binding motif, like that of chicken pT231 antibody (Fig. 4B and C). In huPT0, however, the side chain of ArgH71 interacts with AsnH76 via hydrogen bonding, which results in abolishing the interactions between ArgH71 and the pT231-binding motif residues and thereby disrupting the bowl-like conformation (Fig. 4B and C). Thus, the back mutation of human AsnH76 to chicken SerH76 in huPT9 seems to play a critical role in recovering the pThr-binding conformation through the induction of the correct positioning of ArgH71 toward the VH-CDR2. The critical roles of the position of the H71 FR residue and the conformation of VH-CDR2 via hydrogen bonding have been previously reported [25].

4. Conclusion

Here, we demonstrated the successful humanization of a phosphospecific chicken antibody with the unique pThr-binding motif on VH-CDR2. To our knowledge, our study is the first report on the humanization of antibodies specific to posttranslationally modified protein/peptide antigen, including phosphospecific antibody. Simple transfer of the chicken pT231 antibody CDRs into a homologous human FR template failed to retain the antigen binding activity. Complete restoration of antigen binding specificity and affinity was obtained by the retention of chicken FR amino acids at the four Vernier zone residues of H67, L46, L66, and L71, as well as the H76 residue, the combination of which was efficiently identified by a yeast surface-displayed library with permutations of 11 FR residues potentially affecting CDR loop conformations. Specifically, we demonstrated that back mutation of the H76 residue (Asn to Ser) plays a critical role in maintaining the pThr-binding motif conformation via its allosteric regulation of ArgH71, which closely interacts with ThrH52 and SerH52a residues on VH-CDR2 to form the unique pThr-binding bowl conformation.

In conclusion, our humanization approach of CDR grafting plus permutations of FR residues by combinatorial library screening can be applied to non-human antibodies containing unique binding motif on CDRs specific to posttranslationally modified epitopes. The successfully humanized antibodies in this study can be used for diagnostic and therapeutic purposes associated with the pT231 tau

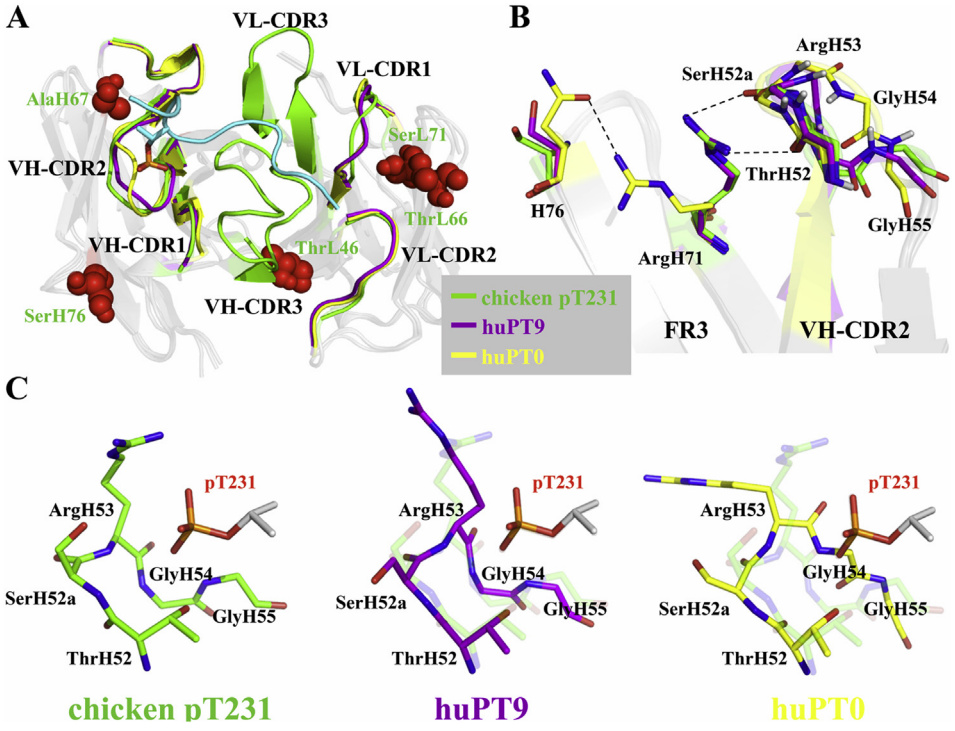

Fig. 4. Structural analysis of the pThr-binding motif on VH-CDR2. (A) Superposition of homology-modeled huPT9 and huPT0 Fv structures with the crystal structure of chicken pT231 antibody (PDB ID: 4GLR) [5]. The cartoon representations of the VH and VL domains of the antibodies are displayed with highlights of the CDRs of each antibody in the indicated color. Five critical FR residues for the recovery of antigen binding activity are highlighted as a red sphere model. The structures of VH-CDR3 of huPT0 and huPT9 were omitted in the imposed structure due to limitations in the loop prediction. (B) Close-up view showing the different conformations of VH-CDR2 due to the differing positions of Arg71H residues between the antibodies. Details are described in the text. (C) Structural comparison of the three antibodies for the pThr-binding motif with the phosphate group of pT231 peptide shown. The pThr-binding motif of huPT9, but not huPT0, forms a bowl-like conformation for the docking of the phosphate group that is comparable to that of the chicken pT231 antibody. The images were generated using PyMol software (DeLano Scientific LLC). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protein [26] and also serve as a valuable template for generating a pThr-peptide-focused human antibody library to bypass animal immunization [6].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.086.

Transparency document

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