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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.

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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

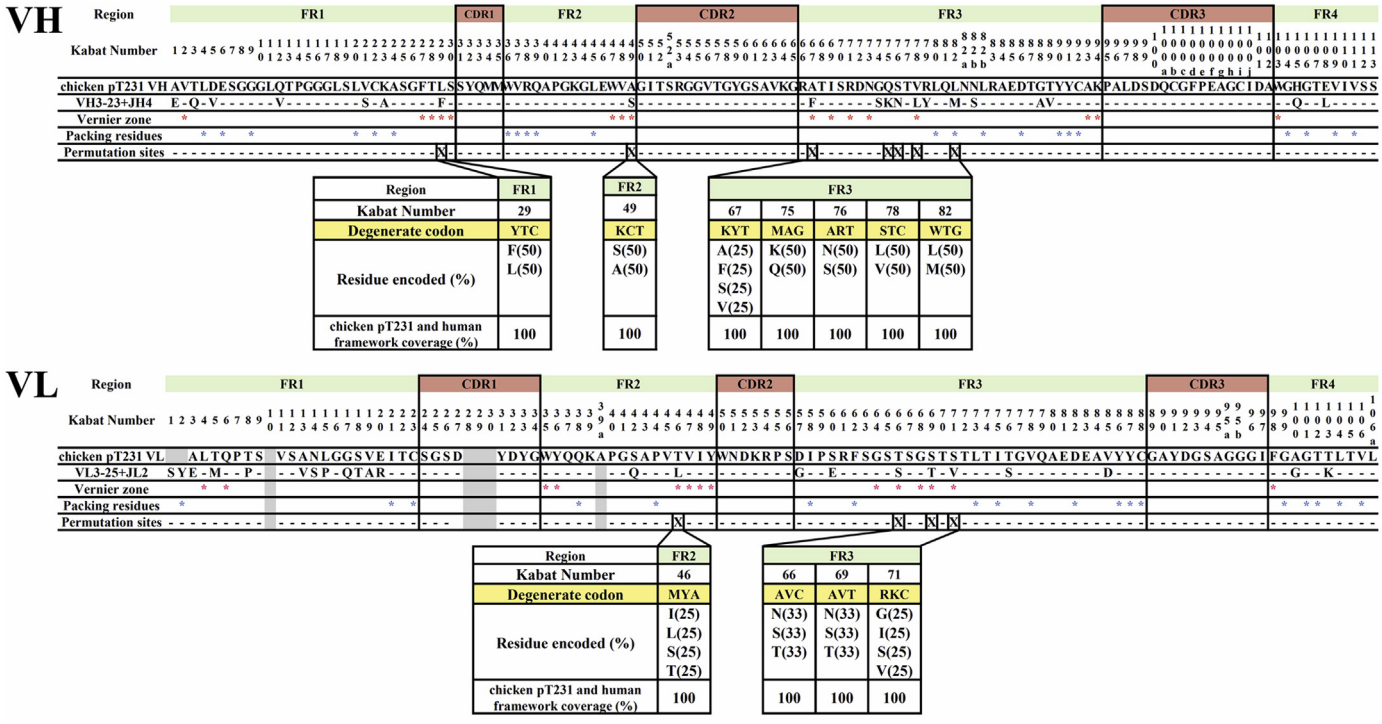

Fig. 1. Amino acid sequence alignment between the VH and VL domains of the chicken pT231 antibody and chosen human germline template, and the library construction strategy involving permutations of the indicated FR residues. The germline VH3-23 and VL3-25 showing the closest sequence homology to the 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.

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