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Next-generation antibodies for post-translational modifications

Takamitsu Hattori¹, Shohei Koide^{1,2,3}

¹Perlmutter Cancer Center, New York University Langone Medical Center, New York, NY, 10016

²Department of Biochemistry and Molecular Pharmacology New York University School of Medicine, New York, NY, 10016

³Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois, 60637

Abstract

Despite increasing demands for antibodies to post-translational modifications (PTMs), fundamental difficulties in molecular recognition of PTMs hinder the generation of highly functional anti-PTM antibodies using conventional methods. Recently, advanced approaches in protein engineering and design that have been established for biologics development were applied to successfully generating highly functional anti-PTM antibodies. Furthermore, structural analyses of anti-PTM antibodies revealed unprecedented binding modes that substantially increased the antigen-binding surface. These features deepen the understanding of mechanisms underlying specific recognition of PTMs, which may lead to more effective approaches for generating anti-PTM antibodies with exquisite specificity and high affinity.

Keywords

antibody engineering; protein design; molecular recognition; directed evolution; antibody-antigen interaction

Introduction

Post-translational modifications (PTMs) of proteins, including methylation, acetylation, phosphorylation, glycosylation and ubiquitination, expand chemical properties of amino acid side chains and hence of entire proteins. PTMs play key roles in many biological processes, such as cell signaling, regulation of gene transcription, protein degradation and cell-cell adhesion [1–4], and their dysregulation is associated with diseases such as cancer [5–8]. Thus, identification and quantification of PTMs are critical for a fundamental understanding of cell biology and for elucidating disease mechanisms. Such methods may also be useful for diagnosis. Characterization of PTMs heavily relies on proteomics analyses where

Corresponding authors: Takamitsu Hattori (Takamitsu.Hattori@nyumc.org) and Shohei Koide (Shohei.Koide@nyumc.org).

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antibodies are an essential component for detecting and enriching PTMs [9,10]. Consequently, there have been high demand for anti-PTM antibodies, but the development of highly specific and potent antibodies to PTMs has been challenging. Chemical moieties of PTMs attached on a protein are usually minute, and differences among PTMs are subtle (e.g. mono- and di-methylation). In addition, specific recognition of either both PTM and sequence surrounding the PTM or a PTM regardless of the surrounding sequence is often required. Thus, these requirements present formidable challenges in molecular recognition.

The level of difficulties in generating highly functional antibodies to PTMs can be compared to that for therapeutic antibodies. Therapeutic antibodies must be selective to their cognate antigens with minimal cross-reactivity to off-targets such as homologs, various members of a protein family, as well as any proteins in the serum and on the cell surface. High affinity is also necessary for high efficacy. In addition, their specificity and efficacy need to be validated through rigorous tests in vitro and in vivo. Since the invention of the hybridoma technology that enables production of monoclonal antibodies after animal immunization [11], methods for generating therapeutic antibodies have been developed continuously. Advances in recombinant DNA technologies enabled us to identify antibodies from large libraries, including naïve [12,13], immunized [14] and synthetic [15,16] libraries, by using molecular display techniques such as phage display [17,18], yeast display [19] and ribosome display [20]. Antibodies identified by these methods can serve as “lead antibodies” that can be subjected to further improvement. The in vitro selection technology combined with creative designs of antibody libraries is particularly powerful for improving affinity and specificity. For instance, affinity maturation of lead antibodies is a common step for increasing their efficacy and reducing dosage of antibodies [21,22]. Similarly, maturation can improve specificity of lead antibodies to their cognate targets among homologs [23,24], and also conversely in broadening specificity to improve neutralization potency [25,26]. Such iterative improvement has enabled antibodies to achieve exquisite specificity and high affinity, and thus this approach has become standard in developing therapeutic antibodies.

Despite the high level of the challenge in developing anti-PTM antibodies is similar to that in developing therapeutic antibodies, most antibodies to PTMs have been generated by the conventional animal immunization without further improvement. This is probably because of multiple factors: limited revenues generated by reagent antibodies present low economic incentives to make large investment in producing high-quality antibodies; many antibodies to PTMs have been developed by end users who do not have access to expertise in antibody engineering. The animal immunization methods indeed have produced high-quality antibodies [27–29]. However, such antibodies are exceptions rather than the norm. Not surprisingly, the specificity problem of available antibodies to PTMs is increasingly recognized in many fields. For instance, more than 25% of over 200 commercial antibodies to histone PTMs failed in a specificity test [30]. Significant variability in affinity and specificity exist among different antibody products to the same PTM and also among different lots of one product [28,31]. Non-specific binding of anti-phosphorylation antibodies has been documented [32,33]. These and many other reports confirm that the identification of highly specific antibodies to PTMs by animal immunization is challenging, as expected from the underlying difficulties in molecular recognition.

In addition to the performance (i.e. specificity and affinity) problem of antibodies, most of current antibodies for research, including antibodies to PTMs, are polyclonal antibodies that cannot be reproduced. Non-renewable reagents such as polyclonal antibodies present serious impediment in obtaining reproducible and reliable data. In the past the antibody bottleneck may have been a localized problem in which a quality problem of an antibody affected a small number of researchers. However, recent advances in genomics and proteomics have enabled large-scale, comprehensive studies that produce large datasets intended as community resources. Thus, the antibody bottleneck has become a worldwide problem impacting many researchers [34]. For example, datasets in the genome-wide histone PTM analysis using two distinct antibodies to the same PTM mark show inconsistent profiles [30], making the entire database of little use. As these experiments are often performed in the context of large-scale projects, the needs for highly functional and renewable antibodies only increase.

The demonstrated successes of iterative improvement approaches in developing therapeutic antibodies strongly suggest the applicability of these same methods to antibodies to PTMs. Recently, several groups have indeed applied this powerful method to generate “next-generation” anti-PTM antibodies, resulted in encouraging outcomes. These next-generation antibodies are recombinant and monoclonal by definition. Their renewability and well-characterized features can eliminate a major bottleneck in producing consistent results. In this review, we highlight studies in developing next-generation antibodies to PTMs, where careful designs of libraries rooted in the knowledge of antibody structure and function are a key to successes, and the combination of structure-guided design and iterative improvement have facilitated generation of highly functional antibodies. Equally important, structural studies of these antibodies give new insights and guide for the generation of antibodies to PTMs.

Anti-PTM antibodies via iterative improvement

Iterative improvement is primarily composed of 1) identification of a lead antibody, 2) elucidation of the structure-function relationship of the lead antibody, 3) design of next-generation antibody libraries and 4) identification of antibodies with improved properties. Steps 2–4 are repeated until antibodies with desired properties are generated (Figure 1a).

In several studies, lead antibodies to PTMs were identified from naïve or synthetic antibody libraries [31,35,36]. The inclusion of negative selection against an appropriate decoy antigen seems to be a key step for identifying antibodies specific to PTMs. However, these antibodies straight from a naïve library exhibit moderate specificity and/or affinity. Level of specificity and affinity of these antibodies are probably similar to typical antibodies generated by animal immunization, suggesting that identification of highly specific antibodies to PTMs is challenging regardless of technology used. However, in iterative engineering approaches, identified antibodies with suboptimal performance are used as the starting point for the next engineering steps, rather than restarting immunization using different animals.

Effective design of the second-generation library is critical for rapid improvement of a lead antibody. Extensive studies of antibody sequences and structures have established modular architecture of IgG wherein the fragment of antigen binding (Fab) recognizes an antigen (Figure 1b). An antigen binding site within Fab is composed of six hypervariable loops, called complementarity-determining regions (CDRs), located within the variable domain of the heavy chain (VH) and that of the light chain (VL) (Figure 1c). Because CDRs are primarily important for the antigen recognition and introducing mutations in the antibody framework may impact the structural integrity, next-generation libraries are commonly constructed by introducing mutations at residues within CDRs. However, the CDRs include approximately 50 residues in total and only a finite combinations of mutations can be experimentally tested in a reasonable time frame, one needs to be smart about how to explore the sequence-function relationship of the lead antibody toward improving its function. Clearly, one wishes to avoid mutating critically important residues to a nonfunctional amino acid. Similarly, mutating a position distant from the bound antigen would not be productive. Thus, undertakings solely based on blind search, such as those based on error-prone PCR, are unlikely to be effective. The challenge of identifying sites for mutation may be particularly acute in antibodies to PTMs, because a small number of residues interact with a small antigen such as a peptide harboring a PTM.

Scanning mutagenesis methods including alanine scanning [37], shotgun scanning [38] and deep mutational scanning [39] have been developed for analyzing this relationship. Hattori et al identified key residues important for antigen recognition by shotgun scanning mutagenesis of most CDR residues of a lead antibody to histone trimethylated Lys. Based on this information, a custom library was designed where only a subset of CDR residues of the lead antibody were diversified, from which final antibodies with exquisite specificity and high affinity to histone trimethylated Lys were successfully developed [31]. A conceptually similar strategy has been employed to develop antibodies specific to poly-ubiquitin linkages, where residues important for the antigen binding were deduced based on the sequence diversity among first-generation antibodies, and a second-generation library was constructed by introducing additional diversity at a subset of CDR residues of a lead antibody [35]. Another design of the second-generation library to linear poly-ubiquitin has been reported, in which each residue in each CDR was randomized with 20 amino acids and the optimal set of mutations were identified from these comprehensive libraries [36]. In all these cases, extensive analyses of the sequence-function relationship of lead antibodies provided critical information for effectively designing next-generation libraries, from which highly functional antibodies are identified. Together, these examples demonstrate the feasibility of identifying high-performance anti-PTM antibodies via iterative improvement.

Anti-PTM antibodies via structure-guided design

Whereas the primary structure (i.e. sequence) of antibodies are a rich source of information, clearly the 3D structures of antibody-antigen complexes provide much more in-depth information of the mechanisms underlying antigen recognition. Thus, 3D structures serve as a powerful guide for designing and engineering antibodies [40,41], as well as for designing next-generation antibody libraries [42] (Figure 1a). In the development of anti-PTM antibodies, structural-guided design were used for enhancing functions of HIV-neutralizing

antibodies [43]. In this study, a binding motif of the lead antibody to N-linked-glycan of gp120 was identified by structural analysis, and then this binding motif was grafted into another lead antibody that recognizes a separate N-linked-glycan of gp120. Consequently, this “chimera” antibody, by recognizing two distinct N-glycans of gp120 synergistically, exhibits higher affinity and enhanced neutralizing activity compared with the two lead antibodies [43]. Although recognition of each glycan motif was not improved, having two binding sites for two distinct motifs within a single antibody significantly improved its function. Similar observation was found in antibodies to histone trimethylated peptides where two distinct binding pockets respectively recognize a PTM and the histone N-terminus [44]. These examples support the effectiveness of exploiting multivalent interactions to enhance their specificity and affinity (see below for additional discussion).

Structure-guided design has been exploited for designing lead antibodies to PTMs (Figure 1a). A lead antibody exhibiting specific recognition of phosphorylated Ser or Thr was developed by building the phospho-specific binding pocket into CDRs, which was inspired by the observation of a common anion binding pocket in natural proteins [45]. This approach is conceptually similar to “loop grafting” where CDR residues are replaced with residues forming a binding site in a natural protein [46,47]. Although antibodies have been designed based on the mechanistic knowledge of how proteins recognize their cognate ligands, transferring a binding function from natural proteins to an antibody is challenging because such grafted motifs often lose the proper structural context that support the productive orientation of critical residues [48]. Koerber et al conquered this challenge by iterative improvement using structural information and directed evolution. Remarkably, the crystal structure of the antibody-phosphopeptide complex revealed that the lead antibody recognized PTM as designed. Final antibodies that recognize the intended phospho peptide, albeit moderate affinity, were generated by an additional round of iterative improvement guided by the knowledge of the general structural basis for antibody-antigen interaction. This study clearly demonstrates that a structure-guided strategy can further enhance the effectiveness of iterative improvement for generating highly functional antibodies to PTMs.

Instead of exploiting the common PTM binding motifs, Yasui et al designed highly specific binders, although they are not antibodies, to phosphotyrosine by exploiting the naturally occurring protein itself [49]. Recombinantly prepared natural proteins can be used as affinity reagents, however their specificity and affinity are generally not sufficiently high for applications. To overcome this challenge, the authors used the affinity clamping technology where evolvable “monobody”, an alternative antibody scaffold, was attached to the peptide-binding module [50]. A large combinatorial library was constructed by diversifying surface exposed loops in the monobody, from which binders exhibited much higher sequence specificity than the peptide binding module were successfully generated. This example shows that iterative improvement using structure-guided design has enabled to generate highly specific designer proteins to challenging antigens, illustrating broad applicability of this approach for generating binders to PTMs.

Novel mechanisms underlying PTM recognition by next-generation antibodies

Our understanding of binding mechanisms of antibodies to PTMs have been limited, because of the limited availability of crystal structures of antibody-PTM complexes and systematic mutagenesis studies, which in turn limits opportunities for applying structure-guided design to iterative improvement of anti-PTM antibodies. Recently, several groups successfully determined the crystal structures of antibody-PTM complexes that reveal novel binding mechanisms. Studies revealed that the topography of the antigen-binding site, which is controlled primarily by the length of CDRs, is distinct for different class of antigens [51,52]. Antibodies to proteins have relatively flat binding surfaces, whereas concave shapes are seen among antibodies to small antigens. Thus, antibodies to peptides or small molecules generate large antigen interacting surfaces by creating a deep cleft in the antigen-binding site. The crystal structure of the antibody-phosphopeptide complex indeed showed the expected binding mode where the phosphopeptide was bound into the concave surface of the antigen-binding site (Figure 2) [53]. PTMs are installed and removed by enzymes, and thus modification sites must be accessible by them. Indeed, they are often found in disordered regions [54]. One might expect that the path to success starts with shaping the antigen-binding site topography according to this rule. However, in our unpublished research, a library constructed based on the general features of antibodies that present a deep cleft in the antigen-binding site did not generate anti-PTM antibodies, supporting the difficulties in designing anti-PTM antibodies based on general knowledge of antibody-antigen interactions.

A new binding mode was found in anti-PTM antibodies that does not follow the common paradigm in antibody-antigen recognition [44]. Antibody fragments such as Fab or Fv contains a single antigen-binding site, and the 1:1 stoichiometry of the antibody-antigen recognition is highly preserved. Strikingly, the crystal structures of antibodies in complex with histone peptides harboring trimethylated Lys show two antigen-binding sites cooperatively sandwich one antigen by forming head-to-head homodimers, a binding mode dubbed “antigen clasp” (Figure 2). Although there is a deep pocket that recognizes the trimethylated Lys within the antigen-binding sites, the overall topography of the antigen-binding site of antibodies is flat. An exceptionally large antigen-binding surface is generated between two flat surfaces. Eight CDRs from two Fab molecules of the clasp antibody to trimethylated Lys 9 on histone H3 make contacts with the single antigen peptide. Another clasp antibody utilizes all twelve CDRs to recognize two copies of a histone peptide harboring trimethylated Lys at position 4 of histone H3. These extensive interactions most likely contribute to the exquisite specificity and high affinity of these antibodies. Interestingly, antigen clasp is similar to the binding mode of affinity clamps as described above [49,50]. In the crystal structures, the target peptides are bound in the clefts created at the interface between a monobody module and the peptide binding module as originally designed, and thus affinity clamps achieved high sequence specificity by expanding the recognition surface. The convergence of the binding modes between antigen-clasp antibodies that are the product of directed evolution and affinity clamps that are the product of structure-guided design suggests that sandwiching an antigen is among the ideal binding

modes for recognizing small antigens, including PTMs, peptides and small compounds. New high-performance capture reagents may be designed by exploiting this principle.

Another novel binding mechanism was found in the HIV-neutralizing antibody, 2G12, where additional antigen binding surfaces are created between two Fab units, surfaces that are not commonly involved in antigen recognition [55]. The conventional antigen-binding site of 2G12 Fab recognizes its antigen with the conventional 1:1 stoichiometry. However, 2G12 Fab forms a stable, domain-swapped homodimer. As a consequence, the dimer contains two antigen-binding sites within it. Strikingly, this dimerization results in the formation of new clefts between two VH domains that bind to the antigen (Figure 2). Consequently, the 2G12 Fab dimer simultaneously binds four copies of a glycan moiety within the conserved glycan cluster of gp120 surface. Such multivalent interactions between an antibody and an antigen lead to an enhancement of the effective affinity, often referred to as the avidity effect. A phage-display system has been established for such domain-exchanged Fab molecules, which has produced promising outcomes [56]. Together, these studies reveal unpredicted binding mechanisms underlying PTMs recognition by antibodies. The current strategies in designing and engineering antibodies are heavily biased by the existing paradigm of antigen-antibody recognition. These and additional novel binding modes gained from structural studies of anti-PTM antibodies will provide important guidelines for designing next-generation antibodies to PTMs.

Conclusions and prospects

The studies highlighted here clearly demonstrate that iterative improvement can be applied to producing anti-PTM antibodies with exquisite specificity and high affinity and that the effectiveness of this approach is further enhanced by structure-guided design. The iterative processes of generating highly functional antibodies and elucidating their binding mechanisms will not only improve the technology for generating antibodies to PTMs but also lead to new types of antibodies that are particularly suited for recognizing PTMs.

Although technologies for improving affinity and specificity of the lead antibody are well established, identification of lead antibodies to PTMs remains a bottleneck. Whereas an increasing number of new antibody libraries designed specifically toward PTMs, such as the library to phosphopeptide as described above, may become available in the future, existing monoclonal antibodies with moderate specificity and affinity to PTMs may well serve as lead antibodies. Therefore, careful validation and sequencing of such monoclonal antibodies can produce useful resources that potentially accelerates the generation of high-quality antibodies to PTMs, which will in turn broadly benefit the biological and biomedical science community [34].

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- of special interest
- of outstanding interest

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Highlights

1. Generation of high-quality antibodies to PTMs is challenging
2. Iterative engineering produced next-gen anti-PTM antibodies of exceptional quality
3. Structure-guided design enhances the effectiveness of iterative improvement
4. Structures of anti-PTM antibodies reveal novel binding modes
5. Next-gen anti-PTM antibodies will eliminate a reproducibility bottleneck

Figure 1.

Iterative improvement of antibody function. (a) Iterative improvement processes. Lead antibodies are developed by animal immunization, directed evolution or structure-guided design. The subsequent processes of designing next-generation libraries, selection or screening and characterization of candidate antibodies produce the final antibodies with desired properties. (b) IgG architecture. (c) CDRs that create the antigen-binding site. An “antigen view” showing the CDRs.

Figure 2.

Novel binding mechanisms found in anti-PTM antibodies. The AT8 Fab/Tau phosphopeptide complex (PDB entry code 5E2W) [53] (left panels), the 309M3-B Fab/H3K9me3 peptide complex (4YHP) [44] (center panels) and the 2G12 Fab/Man9GlcNAc2 complex (1OP5) [55] (right panels) are shown. Top and bottom panels show crystal structures and their schematic structures, respectively. The heavy and light chains are shown in blue and light blue, respectively, and those in the second Fab (the dimerization partner) are shown in green and light green, respectively. Antigens are shown in gold.