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Next-generation antibodies for post-translational modifications

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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.

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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 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-methylation 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.

Although 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 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 a 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 these powerful methods to generate 'next-generation' anti-PTM antibodies, which 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 guidance 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 an antibody with desired properties is 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 primarily 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