

Protein microarrays: A new tool for profiling antibody cross-reactivity

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Abstract. Antibody cross-reactivity can compromise interpretation of experiments and derail therapeutic antibody development. Standard techniques such as immunohistochemistry or Western analysis provide important but often inadequate approaches to assess antibody specificity. Protein microarrays are providing a new approach to rapidly characterize antibody cross-reactivity against 1,000s of proteins simultaneously. This review will focus on reported examples of antibody cross-reactivity, methods used to characterize them, and the recent development and use of protein microarrays for assessing antibody specificity.

Keywords: Antibody cross-reactivity, protein microarray, antibody specificity

1. Introduction

Antibodies have been successfully exploited for applications ranging from basic research to the clinic. In the research lab, antibodies are commonly used tools for affinity purification, co-immunoprecipitation, quantitation and localization of proteins within tissues or cells. In the clinical setting, antibodies are used to quantitate protein levels for diagnostic purposes, and their ability to either inhibit biological action or target specific cells for destruction forms the basis of their use as therapeutics. The success of each of these applications is largely due to, and contingent upon, the high affinity and specificity of antibodies for their antigen targets. Even though specificity is a hallmark of antibodies, cross-reactivity is not infrequently observed. Unrecognized, such cross-reactivity can have adverse consequences. The ability to assess and identify antibody cross-reactivity is an important but often inadequately addressed requirement for both research and clinical applications.

The importance of antibody cross-reactivity characterization for research purposes is obvious, as results derived from experiments using poorly characterized

antibodies can compromise or even invalidate interpretation of experiments. The importance of cross-reactivity screening for clinical applications is underscored in the FDA 'Points to Consider' publication [1], developed for manufacturers of monoclonal antibody products for human use. Recognizing that 'non-target tissue binding may have serious consequences,' the publication suggests screening cells or tissues for cross-reactivity using immunocytochemistry or immunohistochemistry. However, the FDA also suggests that 'appropriate newer technologies should be employed as they become available and validated'. Here we examine a new technology that characterizes antibody cross-reactivity; antibody profiling on protein microarrays. To put this in proper context, however, we will first review a number of documented examples of antibody cross-reactivity and discuss current standard approaches to antibody cross-reactivity profiling.

2. Antibody cross-reactivity

The literature is replete with examples of cross-reactive antibodies. These examples have been identified both through the course of experimentation and through studies intentionally designed to characterize antibody specificity. Although in some cases researchers were able to identify potential sources for the

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observed cross-reactivity, the identity of cross-reactive proteins for the most part remains almost impossible to predict. Antibody specificity profiling therefore remains an important area of ongoing investigation.

Not surprisingly, instances of antibody cross-reactivity can have substantial impact on data interpretation, particularly if cross-reactive proteins are expressed in the same cells or tissues. For instance, while characterizing the epitope of a monoclonal antibody against purified amyloid plaque cores, Tamaoka et al. observed reactivity to a 35 kDa protein in Western blots that was revealed by protein sequencing to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [2]. Both amyloid β -protein and GAPDH were also recognized in a dot-binding assay. Given the ubiquitous nature of GAPDH, the cross-reactivity of this antibody can obviously challenge data interpretation. In another example, Liu et al. [3], demonstrated that C219, a widely used monoclonal antibody directed towards p170^{mdr-1}, also reacts with p185^{c-erbB2}. This cross-reactivity was demonstrated by both Western blotting and immunohistochemical analyses and could be explained by epitope sequence similarity between p170^{mdr-1} (VVQVALD) and p185^{c-erbB2} (VVQGNLE). Since these proteins are known to be co-expressed under certain conditions, the cross-reactivity could lead to false positive results. In a third example, antibodies directed towards commercially available phospho-MKK1/MKK2 (MAP kinase kinase-1 and -2) have been reported to recognize a ~35 kDa protein [4,5] recently identified as a phosphorylated nucleophosmin [6]. Because nucleophosmin is involved in centrosome duplication and nucleolar assembly and transport, the authors advise caution when using such antibodies to examine the regulation of MKK1/MKK2 during mitotic transitions. Recently, Pötgens et al. used 2D electrophoresis and mass spectrometry to identify cytokeratin 18 as a protein cross-reactive with a commercially available antibody against CD133 [7]. Because both CD133 and cytokeratins are present in epithelia, interpretation of immunohistochemistry results using this antibody must be interpreted with caution. In some cases, cross-reactivity can even be 'induced' by the processes used to fix cells and tissues. In one study, a monoclonal antibody to vimentin was observed to cross-react with an unrelated protein only when glutaraldehyde was used as the fixative [8].

When cross-reactivity is uncovered in antibodies that have been used to assign cellular or tissue distribution to proteins, or to explore the biological roles of proteins, a reassessment of data may be required. Aoyama

et al., noticed that the immunohistochemical distribution of transforming growth factor- α protein (TGF- α) appeared to vary depending on the antibody used [9]. Suspecting poor specificity as a cause, Western analysis was used to demonstrate that a human antibody cross-reacted with non-TGF- α proteins, as well as mouse carbonic anhydrase II. Because the human antibody did not recognize rodent TGF- α , but did in fact interact with other proteins, the authors called for a re-examination of TGF- α distribution in rodent tissues. A study of the specificity of a monoclonal antibody generated using a peptide sequence derived from the human immunodeficiency virus (HIV) type-1 transmembrane protein gp41 revealed cross-reactivity with an 100 kDa antigen expressed by astrocytes in human brain tissue [10], later identified as α -actinin [11]. In another example, ELISA competition assays with acetylated peptides were used to select for antibodies directed against histone acetylation sites [12–16]. However, subsequent investigations have indicated that such approaches are insufficient because antibodies that displayed specificity in ELISA's apparently interact with both the wild-type and mutant proteins not expected to be acetylated. Therefore, such ELISA-based approaches for assessment of antibody specificity may not adequately address antibody specificity.

Several studies have been performed for the specific purpose of characterizing antibody sets for cross-reactivity. For instance, Coers et al. investigated the specificity of ten commercially available rat polyclonal anti-NOS (nitric oxide synthase) antibodies [17]. While most antibodies behaved as anticipated, several of the antibodies failed to react with the expected cell populations in certain species, or reacted unexpectedly. Additionally, several antibodies raised against the same NOS isoform did not perform well with identical cell populations. Finally, Western analysis rarely detected proteins of the expected molecular weights using tissue homogenates. The authors suggest that straightforward immunohistological and immunoblotting techniques may be insufficient for the demonstration of specificity. In the first multi-center international collaborative study of its kind, monoclonal antibodies directed towards various subclasses and allotypes of IgA were evaluated for immunoreactivity and specificity by nine laboratories [18]. Although the putative specificities were usually confirmed by study participants, several inconsistencies were observed. In another study, the specificity of seven commercially available anti-p53 monoclonal antibodies was evaluated with Western blotting, immunohistochemistry, and confocal laser



Fig. 1. α -GST image of a human protein microarray. Image was generated from a protein microarray made with $\sim 2,000$ different N'-GST fusions of fully sequenced human open reading frames expressed in insect cells. The array was probed with a fluorescent α -GST antibody and scanned on a fluorescent microarray scanner.

scanning microscopy to evaluate their utility for multi-parameter flow cytometry [19]. Of the three antibodies which demonstrated positive Western results, one also reacted with an unidentified ~ 80 kDa protein in all cell lines tested, despite being described as 'wild-type p53 specific.' Clearly, antibody cross-reactivity is very prevalent despite marketing efforts that suggest otherwise.

While a lack of antibody specificity can sometimes be explained by sequence similarity between cross-reactive proteins, in many instances antibodies may recognize a non-linear conformational protein epitope. For example, an antibody directed against a synthetic peptide derived from the polyoma virus middle T-antigen was found to cross react with a major cytoskeletal protein apparently unrelated to the cognate antigen [20]. In another example a monoclonal multi-drug transporter antibody was found to cross-react with an unrelated yet abundant skeletal muscle myosin [21]. Immunoprecipitation studies with a commonly used monoclonal antibody (SW5) directed against the human La protein led to the identification of an unrelated 80 kDa cross-reactive protein, EEA2 (early endosome antigen 2) [22]. The cross-reactive epitope of EEA2 was shown to reside primarily within an alpha-helical region, while SW5 recognizes a conformational epitope of La [23]. Interestingly, previous studies with human La protein antibodies have been reported to cross-react with laminin [23], although linear epitopes were involved in this case [24]. The type of cross-reactivity is important to bear in mind when evaluating experimental approaches to characterize antibody specificity. While cross-reactivity is easy to visualize on Westerns, such an approach has potential to misrepresent antibody

specificity because of the highly denaturing conditions in immunoblotting assays.

The large number of cross-reactive antibodies is certainly cause for concern. However, perhaps more concerning are antibodies in current use with unrecognized cross-reactivity. Literature reports of cross-reactivity possibly represent the tip of a very large 'iceberg.'

3. Approaches to specificity profiling

The potential for cross-reactivity is widely acknowledged, and the resulting need for specificity profiling is well understood. Surprisingly, there are no universally accepted 'standards' for assessing antibody cross-reactivity. However, there are certainly numerous approaches, the most common being Western blotting [25–36], immunocytochemistry or immunohistochemistry. These approaches may be used alone [25–31] or in combination with alternate methods which have not necessarily been validated for characterizing antibody specificity [34,35,37–43].

Competitive ELISA [44] is another approach whereby antibody specificity is addressed by assessing reactivity against proteins related to the target antigen [33, 45,46]. Immunofluorescence [37,46–48], especially in combination with flow cytometry, is often used to show reactivity towards expected cell types and not against negative controls. In many cases these techniques have been combined with other methods. For instance, immunofluorescence, Western blotting and immunohistochemistry have been used to discriminate between transfected and non-transfected cell lines, thus demonstrating specificity [35,42,43,47,49]. Alternatively, the

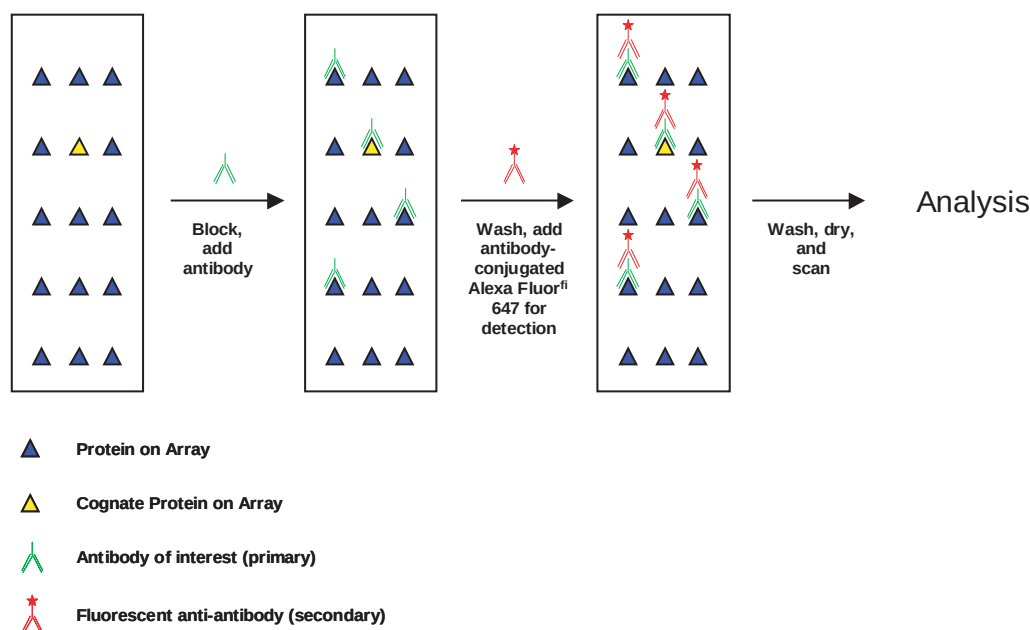


Fig. 2. Schematic diagram of antibody cross-reactivity profiling protocol. In this example, the antibody being tested binds to both its' cognate antigen (yellow triangle) as well as additional proteins (blue triangles). After blocking slides, an appropriate dilution of the antibody of interest is incubated with the microarray. Then, after washing, slides are incubated with a fluorescent secondary antibody, washed, dried and scanned on a fluorescent slide scanner. After image processing and digitization, reactive proteins can be visually identified.

use of genetic knockouts [35] or knockdowns have been employed. Antigen site-directed mutagenesis has been combined with immunoprecipitation, Western blotting and/or cell surface staining to demonstrate antibody specificity for antibodies designed to recognize site-specific post-translational modifications [50–52].

More recently, mass spectrometry-based approaches for protein identification have provided an additional approach to characterizing antibody specificity. Weiler et al. suggested a generalized approach employing bead-immobilized antibodies to capture antigens from complex mixtures such as synovial fluid and cellular lysates, followed by mass-spectrometric antigen identification [53]. Previous investigations have employed 1– [6] or 2-dimensional [7] polyacrylamide gel electrophoresis followed by mass-spectrometric identification. Although this is a promising approach to profiling antibody specificity, obstacles include technically challenging/time-consuming protocols, expensive equipment, and difficulties in identifying some classes of proteins, such as membrane proteins, as well as antigens present at low concentrations.

Ultimately, it is important to evaluate antibodies under conditions as close to possible as their ultimate application. This is consistent with the observation that antibodies are commonly observed to work well in certain conditions, but not in others. Typically, antibodies

which perform effectively in applications requiring native proteins (immunoprecipitation, immunocytochemistry, therapeutics) often perform poorly in applications which require denatured proteins (Westerns), and *vice versa*. For instance, using antibodies to mouse nerve growth factor- β , Conner and Varon demonstrated that cross-reactivity with related neurotrophins was heavily assay dependent [54], an extension of earlier studies demonstrating cross-reactivities in a variety of applications [55–60]. Unfortunately, many antibodies are evaluated for cross-reactivity in only a single assay system, which is often different from the conditions in which they will eventually be employed.

Antibody cross-reactivity can represent a problem or even an opportunity for discovery, but either way it must be understood. However, standard approaches to studying antibody cross-reactivity may not be sufficient. New technologies, such as protein microarrays, are introducing important capabilities to address this challenge.

4. Protein microarrays for antibody cross-reactivity profiling

In the few years since protein microarrays were introduced to the research community, they have been

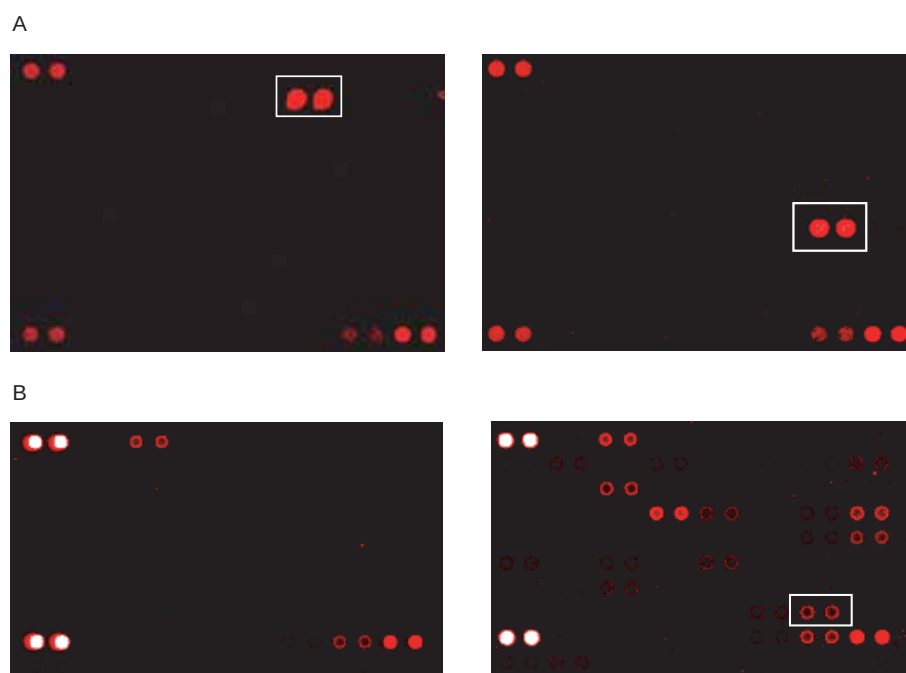


Fig. 3. A. Antibody profiling images of array subsections. Examples of α -Fyn antibody binding to Fyn (left panel, boxed) and to a cross-reactive protein (right panel, boxed). Unidentified spots are controls used as reference points to help assign protein identities to other signals on the array. B. Arrays were profiled with an α -MAPKAPK2 phosphopeptide (SHNpSLTTP) antibody (right panel), with a negative control (no primary antibody) result shown in the left panel. Binding to the cognate protein (MAPKAPK2) is indicated by a white box. Cross-reactive binding is readily apparent by comparison of the negative control (left) to the experimental image (right). A summary of the quantitative analysis of this array profiling is reported in Table 1.

exploited for a variety of applications. An image of a $\sim 2,000$ human protein microarray is shown in Fig. 1. Much like DNA microarrays, arrays of antibodies have been used to profile the concentrations of protein antigens in biological mixtures under varied conditions. Arrays of non-antibody proteins, on the other hand, have been used to characterize enzyme-substrate relationships, investigate molecular interactions, and to profile the specificity of antibodies themselves. A basic protocol for using protein microarrays for antibody cross-reactivity profiling is depicted in Fig. 2.

The use of protein microarrays for antibody cross-reactivity profiling has several advantages over other techniques. To start, the technique is both rapid and simple, typically taking a half day and requiring little specialized training. Unlike many other approaches, this method enables an essentially immediate identification of cross-reactive proteins. Since the concentration of proteins on the arrays are somewhat normalized, the method is often more sensitive than approaches utilizing biological samples. Finally, if the proteins are arrayed in a folded form, the method is capable of identifying structured epitopes.

An early proof-of-concept experiment for antibody cross-reactively profiling was published by Lueking et al. [61]. Microarrays were generated from *E. coli* expression of $\sim 2,400$ human fetal brain cDNA expression clones arrayed onto polyacrylamide-coated glass slides. These microarrays were used to profile the binding specificities of α -glyceraldehyde phosphate dehydrogenase and an α -heat-shock protein. In both cases the antibodies preferentially recognized their cognate antigens, but bound to additional cross-reactive proteins. In a similar study with 96 *Arabidopsis* proteins, only specific antibody binding was observed – not surprising given the small number of proteins tested [62]. A more comprehensive study was reported by Michaud et al. [63], where both polyclonal and monoclonal antibodies to various yeast proteins were profiled against yeast proteome microarrays. As expected, monoclonal antibodies tended to exhibit higher specificity than polyclonal antibodies, though cross-reactivity was still observed.

Some additional examples of antibody cross-reactivity profiling are shown in Fig. 3. Figure 3(A) shows recognition of cognate antigens for 2 antibodies,

Table 1

Normalized ranking of proteins reactive towards an β -MAPKAPK2 phosphopeptide (SHNpSLTTP) antibody. Signals from the protein microarrays (see Fig. 3d) were normalized by the amount of protein estimated on the array. The top ranked protein has the highest signal:protein ratio. The protein towards which the antibody was directed was ranked 17

Rank	Reactive protein
1	OSR1 oxidative-stress responsive 1
2	STK39 serine threonine kinase 39 (STE20/SPS1 homolog, yeast)
3	CSNK2A2 casein kinase 2, alpha prime polypeptide
4	MKNK1 MAP kinase-interacting serine/threonine kinase 1
5	TTN titin
6	BMPR1A: Bone morphogenetic protein receptor, type IA
7	CASK calcium/calmodulin-dependent serine protein kinase (MAGUK family)
8	MKNK2 MAP kinase-interacting serine/threonine kinase 2
9	FLJ23356 hypothetical protein FLJ23356
10	MAP2K2 mitogen-activated protein kinase kinase 2
11	CAMK4 calcium/calmodulin-dependent protein kinase IV
12	CDK2 cyclin-dependent kinase 2
13	PAK2: P21 (CDKN1A)-activated kinase 2
14	STK16 serine/threonine kinase 16
15	SGK2 serum/glucocorticoid regulated kinase 2
16	EPHA4 EphA4
17	MAPKAPK2 mitogen-activated protein kinase-activated protein kinase 2
18	BMX BMX non-receptor tyrosine kinase
19	PIM1 pim-1 oncogene
20	BMPR1B bone morphogenetic protein receptor, type IB
21	IRAK3 interleukin-1 receptor-associated kinase 3
22	CDKL5 cyclin-dependent kinase-like 5
23	BIKE BMP-2 inducible kinase
24	NRBP nuclear receptor binding protein
25	STK22C: Serine/threonine kinase 22C (spermiogenesis associated)
26	EPHB2 EphB2
27	TOPK T-LAK cell-originated protein kinase
28	ABL1 v-abl Abelson murine leukemia viral oncogene homolog 1
29	HRI heme-regulated initiation factor 2-alpha kinase
30	BMPR2 bone morphogenetic protein receptor, type II (serine/threonine kinase)
31	C8FW phosphoprotein regulated by mitogenic pathways
32	AKT1 v-akt murine thymoma viral oncogene homolog 1
33	TGFBR2 transforming growth factor, beta receptor II (70/80 kDa)
34	CDK8: Cyclin-dependent kinase 8
35	AAK1 adaptor-associated kinase 1
36	EPHA3 EphA3
37	SNARK likely ortholog of rat SNF1/AMP-activated protein kinase
38	STK16 serine/threonine kinase 16
39	CAMK1D: Calcium/calmodulin-dependent protein kinase ID
40	TOPK T-LAK cell-originated protein kinase

while recognition of cognate as well as cross-reactive proteins are illustrated in Fig. 3(B). The antibody used to generate Fig. 3(B) is a monoclonal developed towards a phosphopeptide from the kinase MAPKAPK2. As is evident from the figure, this antibody appears relatively nonspecific, recognizing a number of other proteins on the array. The cross-reactive proteins are summarized in Table 1. Interestingly, even after normalization for the amount of protein on the array, the antibody binds to several proteins on the array with higher signals than for the cognate protein itself. Perhaps not surprisingly, several of the cross-reactive proteins are

kinases.

Functional protein microarrays composed of properly folded proteins present structured epitopes. In an elegant example, Alcorer et al. [64] used protein microarrays to demonstrate that seed 2S albumin protein was recognized specifically by an IgE antibody from individuals allergic to Brazilian nuts. Furthermore, protein microarrays of chimeric folded 2S albumin proteins were used to identify the epitope: a structured region in a helix-loop-helix region of the protein. While less well-characterized, cross-reactive epitopes can also be structured, and such structured epitopes can be re-

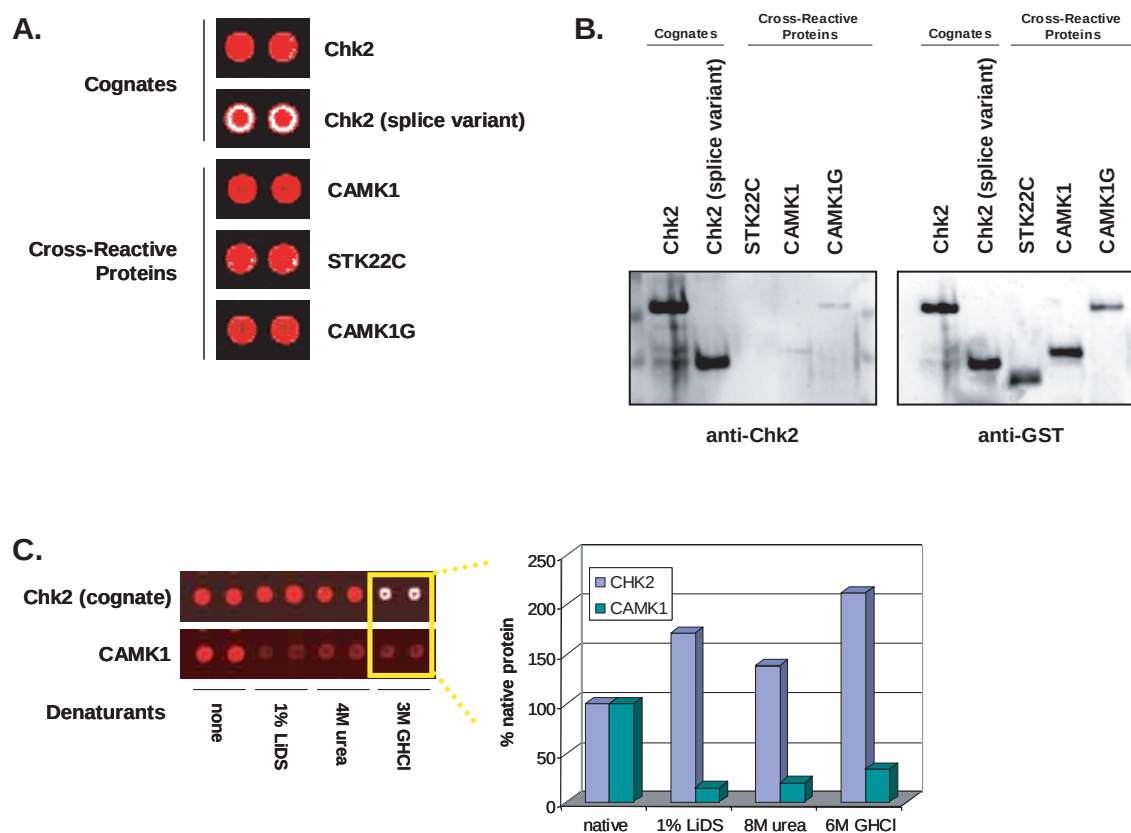


Fig. 4. *Conformational epitope recognition on protein microarrays.* A. Human protein arrays were probed with a monoclonal antibody directed against Chk2. Cross-reactive proteins spotted in duplicate are shown. Red color indicates signal; white color indicates signal saturation. B. Proteins were run on denaturing SDS gels and transferred to nitrocellulose for immunoblotting with anti-Chk2 antibody (left panel) or anti-GST (right panel). Only the cognate protein Chk2 was recognized by the anti-Chk2 antibody in this assay, despite equal loading of all proteins as confirmed by the anti-GST blot. C. Proteins were left untreated, or were treated with chemical denaturants prior to printing. Proteins were printed as duplicate spots and arrays were probed with anti-Chk2 antibody (left panel, top). Chk2 was recognized in both native and denatured form, suggesting antibody recognition of a linear epitope (left panel, top), while CAMK1 was recognized in the native form only, suggesting antibody recognition of a conformational epitope (left panel, bottom). Right panels show quantified array signals.

vealed from antibody profiling screens on protein microarrays as shown in Fig. 4. In this example, a human protein microarray was profiled with an antibody to the kinase Chk2. In addition to observing reactivity with the cognate antigens, three cross-reactive proteins were identified (Fig. 4A). To validate the antibody-antigen interactions, the proteins were probed in western blots with the anti-Chk2 antibody (Fig. 4B). Interestingly, the array identified cross-reactive proteins did not react in the western blot. A simple hypothesis is that the epitopes detected on the protein arrays are conformational. This hypothesis was confirmed by demonstrating that the antibody cross-reactivity is abolished by denaturing the proteins prior to arraying (Fig. 4C). Therefore, protein microarrays are extremely useful tools for profiling and characterizing the nature of antibody-antigen interactions.

5. Conclusion

While a promising approach, the use of protein microarrays for antibody cross-reactivity profiling still presents some challenges. For now, the lack of a complete human proteome microarray prevents a comprehensive specificity analysis. Although a complete human proteome chip may be some time away, highly representative arrays are likely to be developed in the near future. Due to technical challenges, folded membrane proteins may be under-represented, and these represent an important class of proteins for therapeutic antibody development. The ability to control post-translational modifications of microarrayed proteins will be another important issue to address.

Despite the challenges, protein microarrays are already proving to be highly useful tools for antibody

specificity analysis. As the use of antibodies for laboratory and clinical applications expands, protein microarrays provide an important new tool for characterizing their specificity.

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