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Analyzing antibody specificity with whole proteome microarrays

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Although approximately 10,000 antibodies are available from commercial sources, antibody reagents are still unavailable for most proteins[1]. Furthermore, new applications such as antibody arrays[2–5] and monoclonal antibody therapeutics[6,7] have increased the demand for more specific antibodies to reduce cross-reactivity and side effects. An array containing every protein for the relevant organism represents the ideal format for an assay to test antibody specificity, because it allows the simultaneous screening of thousands of proteins for possible cross-reactivity. As an initial test of this approach, we screened 11 polyclonal and monoclonal antibodies to ~5,000 different yeast proteins deposited on a glass slide and found that, in addition to recognizing their cognate proteins, the antibodies cross-reacted with other yeast proteins to varying degrees. Some of the interactions of the antibodies with noncognate proteins could be deduced by alignment of the primary amino acid sequences of the antigens and cross-reactive proteins; however, these interactions could not be predicted *a priori.* Our findings show that proteome array technology has potential to improve antibody design and selection for applications in both medicine and research.

There are a number of potential advantages in using a microarray containing most of the proteins for a given organism to measure the specificity of an antibody to a protein from that organism. In the yeast proteome array used in this study, each protein was immobilized in defined locations on the array. Consequently, if an antibody bound to a protein on the array, the identity of the protein and its sequence were readily available. Because each protein is deposited in roughly equal amounts, the technology affords a screening mechanism that is relatively unbiased in terms of the effect of protein concentration on sensitivity of detection. In addition, proteome arrays allow the screening of large numbers of proteins in both their native and denatured forms, because the arrays can be treated with various denaturants before probing with antibodies. Finally, the sensitivity of antibody detection for all reactive proteins can be assessed simply by probing several identical arrays with a serial dilution of the antibody. These features give protein microarray–based screening of antibody specificity distinct advantages over more commonly used methods of screening. In western blot–based screening, for example, cross-reactive proteins have to be cut from gels and identified by sequencing or mass spectrometry[8]. Antibody screening using western blots of cell lysates or immunohistochemistry of tissue samples is also made more challenging by the much broader range of protein concentrations present, including many proteins that are present only in very low quantities. We have also found that detection of proteins on microarrays is ~100-fold more sensitive than even the most sensitive western blot methods available (data not shown).

Our initial test of proteome microarrays as an antibody screening tool was carried out using the yeast proteome microarray previously described[9,10] and antibodies to yeast proteins. In screening the yeast proteome array, we found that the specificities of the polyclonal and monoclonal antibodies tested ranged considerably. **Figure 1** shows a few examples of the probes carried out in this study, including an example of a probe with one of the monoclonal antibodies that was raised to a non-yeast protein sequence.

On one end of the specificity spectrum were the polyclonal antibodies to Mad2 and Cdc42 peptides, and the monoclonal antibodies to Cox4 and Sed3, which were apparently monospecific for their cognate antigens (**Table 1**). On the other end of the spectrum is the polyclonal antibody to the full-length protein Nap1, which appeared to recognize over 1,000 proteins on the array. The intensity of the microarray signals obtained with the anti-Nap1 polyclonal antibody was correlated with the amount of protein deposited in each spot. Spots of pure glutathione *S*-transferase (GST) on the array gave weaker signal intensities than many GST-fusion proteins present on the array in lower amounts, suggesting that anti-Nap1 did not bind specifically to the GST fusion tags. The correlation of the signal intensities from anti-Nap1 binding with the amount of protein in the spot suggests that the antibody or a component of the polyclonal antibody preparation recognized a common element in the proteins such as a particular amino acid or a simple peptide sequence (*e.g.*, di- or tripeptide).

The majority of the anti-yeast protein antibodies examined in this study exhibited a degree of specificity that was in between that of the nonspecific anti-Nap1 antibody and the four monospecific antibodies.

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Table 1 Antibodies tested on yeast protein microarray

Antibody	Approx. amount of protein on array (pg)	Source of epitope	Immunogen	Antibody type	Antibody concentration	Number of proteins with signal/background >2.0
Ynl021W-Hda1	0.3	Yeast	Peptide$^{20\ a.a.}$	Polyclonal^{a,b}	0.8	8
Ylr113W-Hog1	0.4	Yeast	Peptide$^{20\ a.a.}$	Polyclonal^{a,b}	0.8	2
Yjl164C-Tpk1	1.2	Yeast	Peptide$^{20\ a.a.}$	Polyclonal^{a,b}	0.8	9
Yjl030W-Mad2	2.3	Yeast	Peptide$^{20\ a.a.}$	Polyclonal^{a,b}	0.8	1
Ylr229C-Cdc42	5.2	Yeast	Peptide$^{20\ a.a.}$	Polyclonal^{a}	0.1	1
Ypr120C-Clb5	ND	Yeast	Peptide$^{20\ a.a.}$	Polyclonal^{a,b}	0.1	1
Yjr076C-Cdc11	2.2	Yeast	Protein$^{1–415\ a.a.}$	Polyclonal^{c}	0.04	7
Ykr048C-Nap1	7.2	Yeast	Protein$^{1–417\ a.a.}$	Polyclonal^{c}	0.02	1,770
Ypr183W-Sed3	13.3	Yeast	Protein$^{cytosolic\ domain}$	Monoclonal^{d}	2.0	1
Ygl187C-Cox4	0.9	Yeast	Protein$^{\cdot}$	Monoclonal^{d}	2.0	1
Yor036W-Pep12	4.4	Yeast	Protein$^{C\ terminus}$	Monoclonal^{d}	2.0	4
HA	–	Influenza	Peptide$^{12\ a.a.}$	Monoclonal^{d}	1.0	0
MYC	–	Human	Protein	Monoclonal^{d}	1.0	0
FLAG	–	Synthetic	Peptide	Monoclonal^{d}	2.4	0

The amount of each protein on the array and the number of proteins recognized by each antibody was determined as described in Methods.
[a]Goat IgG isotype. [b]Affinity purified. [c]Rabbit IgG isotype. [d]Mouse IgG isotype. a.a., amino acids; ND, not detected.

For example, the anti-Hda1 polyclonal antibody cross-reacted with seven different yeast proteins. On a western blot, anti-Hda1 recognized its cognate protein Hda1 and only three of the proteins that gave substantial signals on the arrays (**Fig. 2a**); thus, anti-Hda1 may have bound epitopes on these proteins that shared common features. The absence of observable western blot signals for the other four proteins was likely due to insufficient sensitivity of the western or to the presence of a conformation-sensitive epitope that was disrupted in the denaturing gel.

The anti-Hda1 antibody was raised to a 21-amino-acid peptide with the sequence TDGLNNIIEERFEEATDFILD. A comparison of this sequence with that of the seven reactive proteins reveals that the region of highest similarity is entirely contained within the 21-amino-acid sequence of the peptide (**Fig. 2b**). To examine this further, we probed arrays with the antibody in the presence of the immunizing peptide; a peptide of similar length but different sequence was used as a control. The Hda1 blocking peptide, but not the control peptide, inhibited the interaction of anti-Hda1 with its cognate antigen Hda1 as well as with each of the seven cross-reacting proteins (**Fig. 2c**). A sequence identity search against the yeast proteome for matches to the 21-amino-acid immunizing peptide found 86 yeast proteins that had better matches to the immunizing peptide than did any of the proteins identified on the array. Furthermore, only three of the seven cross-reactive proteins observed on the microarray were among the top 1,000 hits to the predicted yeast proteome of approximately 6,300 proteins. Thus, sequence analysis, may be useful in explaining cross-reactivity, but it is evidently insufficient to predict it. These findings suggest that thorough empirical assessments of antibody cross-reactivity such as these will be a necessary feature of any effort to produce large numbers of specific antibodies.

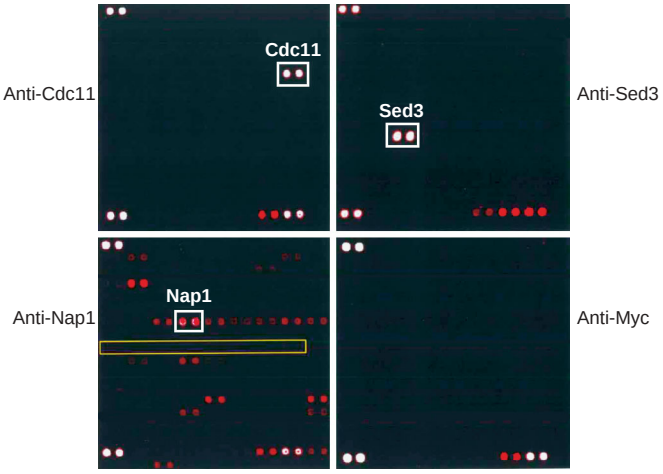

Figure 1 Fluorescent images of antibody probes of the yeast proteome microarray. Subarrays for anti-Cdc11, anti-Nap1 and anti-Sed3 show the antibodies reacting with their cognate proteins, which are boxed in white. For each antibody probe, the signals from cognate proteins ranked among those with highest intensity. For anti-MYC, a typical subarray was chosen to show the absence of signal. The yellow box is drawn around the spots representing the dilution series (0.1–12.8 pg/spot) of pure GST that is printed on each array for quantification purposes. White spots, greater signal intensity; red spots, weaker signal intensity.

Probing the yeast proteome array with polyclonal antibodies to the proteins Cdc11 and Hog1 revealed six noncognate proteins and one noncognate protein, respectively, that cross-reacted with these antibodies. Three out of the six noncognate proteins seen on the array probed with the anti-Cdc11 antibody were also observed on a western blot (see **Supplementary Fig. 1** online). The one protein seen on the array with the anti-Hog1 antibody, however, was not detected on a western blot (data not shown).

In contrast to the above examples, the reactivity observed with the polyclonal antibody to Tpk1 and the monoclonal antibody to Pep12 cannot be accounted for strictly on the basis of an epitope that shares common features. Instead, western blot analysis showed that some of the reacting protein preparations contained small but detectable amounts of the cognate protein. Tpk1 is a protein kinase involved in pseudohyphal growth and Ras signal transduction[11,12]. Substantial signals were observed for eight proteins on the arrays probed with the antibody to this protein. Three of these proteins, Tpk3 (a protein kinase), Tpk2 (a protein kinase) and Bcy1 (a protein kinase inhibitor), have been shown by mass spectroscopy to immunoprecipitate with Tpk1 (ref. 13). Western blot analysis with anti-Tpk1 revealed a protein with the same molecular weight as Tpk1 copurifying with Bcy1 but

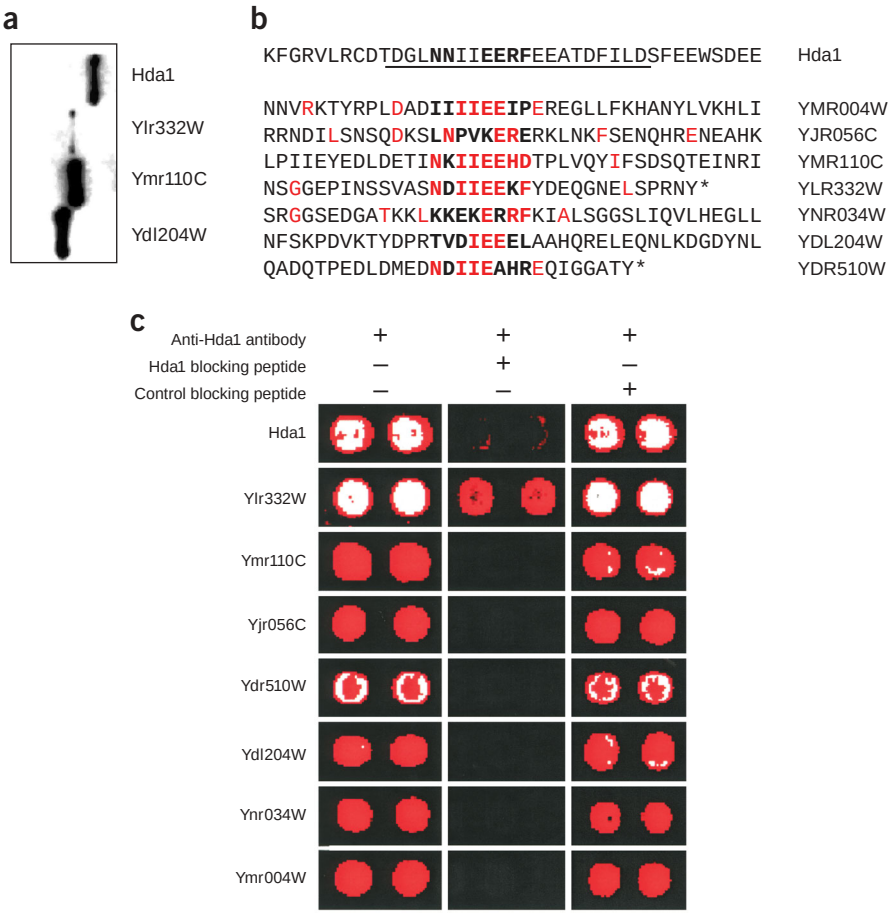

Figure 2 Analysis of anti-Hda1 binding to the yeast proteome microarray. (**a**) Western blot analysis of proteins detected with the anti-Hda1 antibody on the yeast proteome microarray. (**b**) Sequence alignment of the Hda1 immunogenic peptide with best matches from each of the cross-reacting proteins. The immunogenic peptide is underlined in the Hda1 sequence. The eight-amino-acid window with highest maximal sequence identity between all cross-reacting proteins is shown in bold. *, C terminus. (**c**) Peptide inhibition of anti-Hda1 binding on the yeast proteome microarray. Images on the left are from an array that was probed with the anti-Hda1 antibody alone. Images in the middle are from an array that was probed in the presence of the Hda1 immunizing peptide. Images on the right are from an array that was probed in the presence of a peptide with an unrelated sequence.

not with Tpk2 or Tpk3 (see **Supplementary Fig. 2** online). This western blot also showed, however, that anti-Tpk1 bound to the GST fusions of Tpk2 and Tpk3, suggesting that the signals observed for these two proteins on the array with anti-Tpk1 were due to cross-reactivity with related epitopes. These findings are in agreement with microarray-based protein interaction experiments that showed an interaction between Tpk1 and Bcy1, but not between Tpk1 and Tpk2 or Tpk3; Bcy1, however, interacted with Tpk2 and Tpk3 on the array (see **Supplementary Fig. 3** online). To the best of our knowledge, this is the first time that interacting proteins have been purified and detected on microarrays. Furthermore, these results suggest that the copurification of Tpk2 and Tpk3 with Tpk1 (ref. 13) was most likely the result of indirect protein-protein interactions in which Tpk1 interacted with both Tpk2 and Tpk3 through Bcy1.

The anti-Pep12 monoclonal antibody bound to Vti1, Yer010C and Tlg1 in addition to the expected binding to Pep12 (**Fig. 3a**). Interestingly, yeast two-hybrid and affinity purification data indicated that Pep12 interacts with Vti1 (refs. 14,15), and Vti1 has been shown by coimmunoprecipitation to interact with Tlg1 (ref. 16). No references in the literature were found that support an interaction between Pep12 and Yer010C. Consequently, western analysis was carried out to test for the possibility that endogenous Pep12 copurified with one or more of these proteins. The 63-kDa GST-Pep12 fusion protein reacted with both anti-Pep12 and anti-GST antibodies, respectively (**Figs. 3b,c** (lane 1)). A smaller band of ~33 kDa, the expected molecular weight of endogenous Pep12, was recognized by the anti-Pep12 antibody in the Vti1 and Tlg1 preparations, confirming copurification with Pep-12 (**Fig. 3b**). Interactions between Pep12 and Vti1 and between Vti1 and Tlg1, but not between Pep12 and Tlg1, have been reported (refs. 14–16). The copurification of Vti1 and Tlg1 with Pep12 suggests a model in which Tlg1 interacts indirectly with Pep12 through Vti1. In contrast, anti-Pep12 and anti-GST both recognized a 55-kDa protein in the GST-Yer010C sample, suggesting that anti-Pep12 was cross-reacting with a related epitope in this protein.

In addition to testing antibodies for cross-reactivity to proteins present on the yeast proteome array, we also tested antibodies with proteins not present on the array. Monoclonal antibodies anti-HA (an antibody to the influenza hemagglutinin epitope), anti-MYC (an antibody to the Myc epitope), and anti-FLAG (an antibody to the synthetic FLAG epitope[17]) did not produce substantial signals for any protein on the array. Clb5 is a yeast protein that was present in our clone collection, but did not give a detectable GST signal on the printed array. A polyclonal antibody to Clb5, however, detected a protein designated as Yfl045-Sec53. Western blot analysis of this protein showed that both anti-Clb5 and anti-GST bound to a protein at the predicted molecular weight of the GST fusion protein (data not shown), suggesting that Clb5 and Sec53 share a common epitope.

The results showed the utility of whole proteome microarrays for screening antibody specificity. The arrays in combination with western analyses distinguished specific from nonspecific antibodies; furthermore, the identification of the cross-reacting proteins was unambiguously established. This type of information should prove invaluable for correctly interpreting the results of various kinds of biochemical analyses carried out using these antibodies. We expect that similar types of results would be obtained using arrays of proteins from other species, which we are developing, and antibodies to the proteins of these species. In particular, prescreening anti-human antibodies on human proteome microarrays may become an important part of the development of more specific and more effective antibodies for use in the clinic. One unanticipated finding was that antibody screening experiments of this type can be useful for revealing protein-protein interactions and thus can provide new insights into biology.

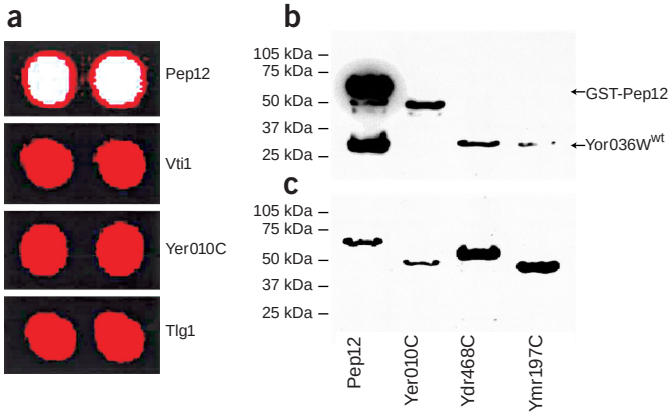

Figure 3 Analysis of anti-Pep12 binding to the yeast proteome microarray. (**a**) Portions of microarray images showing the spots corresponding to the Pep12 protein and the three proteins that gave substantial signals with the anti-Pep12 antibody. (**b**) Western blot probed with anti-Pep12 antibody. (**c**) Western blot probed with anti-GST. Positions of molecular weight standards, GST fusion of Yor036W and endogenous wild-type protein, Yor036W^{wt}, are shown.

METHODS

Preparation of yeast protein microarrays. Yeast proteins were purified as described[9]. Proteins were immobilized on FAST (nitrocellulose pad size, 20 mm × 60 mm) slides (Schleicher & Schuell) by printing each protein in duplicate with a Genemachines Omnigrid arrayer.

Antibodies and probing of microarrays. Antibodies to yeast proteins Hda1, Hog1, Tpk1, Mad2, Cdc42, Clb5, Cdc11 and Nap1 were obtained from Santa Cruz Biotechnology. Antibodies to yeast proteins Sed3, Cox4 and Pep12 were obtained from Molecular Probes. Antibodies to HA and Myc were obtained from Covance, and the antibody to FLAG was obtained from Sigma. Slides were probed with antibodies for 2 h. After washing, secondary antibodies (anti-goat, anti-rabbit or anti-mouse) conjugated to Cy5 (Jackson Immunoresearch Laboratory) were added and incubated for 1 h. Slides were washed, dried and imaged with an Axon 4000B scanner.

For each antibody, the amount of cognate antigen present on the array was determined by probing the array with an anti-GST antibody; the Cy5 signal intensity values of a GST dilution series (0.1–12.8 ng/μl) printed on the array were used to generate a standard curve, which was used to calculate concentrations of GST-fusion proteins on the same array. The antibody concentration used to probe the protein antigen arrays was determined by titering each antibody for maximal reactivity with its cognate protein. We report the number of proteins having a signal-to-background ratio ≥2.0. Local background intensity was determined by the GenePix software. This signal-to-background ratio was at least 3 s.d. over the mean signal-to-background ratio for all of the spots on the array, and was thus considered to be statistically significant.

SDS-PAGE and western analyses. Western blotting was carried out by running purified proteins on SDS-PAGE gels, transferring them to nitrocellulose, probing them with primary antibodies for 2 h and with secondary antibodies conjugated to horseradish peroxidase for 1 h. Femto-reagent (Pierce Biotechnology) was added and images were acquired with an Alpha Innotech Imaging station.

Note: Supplementary information is available on the Nature Biotechnology website.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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