Producing peptide arrays for epitope mapping by intein-mediated protein ligation

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BioTechniques 37:430-443 (September 2004)

Peptide arrays are increasingly used to define antibody epitopes and substrate specificities of protein kinases. Their use is hampered, however, by ineffective and variable binding efficiency of peptides, which often results in low sensitivity and inconsistent results. To overcome these limitations, we have developed a novel method for making arrays of synthetic peptides on various membranes after ligating the peptide substrates to an intein-generated carrier protein. We have conducted screening for optimal carrier proteins by immunoreactivity and direct assessment of binding using a peptide derivatized at a lysine sidechain with fluorescein, CDPEK(fluorescein)DS. Ligation of a synthetic peptide antigen to a carrier protein, HhaI methylase, resulted in an improved retention of peptides and an increased sensitivity of up to 10²-fold in immunoassay- and epitope-scanning experiments. Denaturing the ligation products with 2% sodium dodecyl sulfate (SDS) or an organic solvent (20% methanol) prior to arraying did not significantly affect the immunoreactivity of the HhaI methylase-peptide product. Because the carrier protein dominates the binding of ligation products and contains one peptide reactive site, the amount of peptide arrayed onto the membranes can be effectively normalized. This technique was utilized in the alanine scanning of hemagglutinin (HA) antigen using two monoclonal antibodies, resulting in distinguishing the different antigen epitope profiles. Furthermore, we show that this method can be used to characterize the antibodies that recognize phosphorylated peptides. This novel approach allows for synthetic peptides to be uniformly arrayed onto membranes, compatible with a variety of applications.

INTRODUCTION

Synthetic peptide arrays are powerful tools for characterizing antibodies raised against protein antigens. For epitope mapping, an array is made from a library of short, overlapping peptides that span the antigenic protein sequence (1–3). The antigenic determinant recognized by a monoclonal antibody can then be defined by probing this array with the antibody. An epitope can be defined more precisely by constructing an array in which each epitope residue is substituted with either alanine (alanine scanning) or other amino acids to test which residue’s contribution to antibody binding and to determine which substitutions affect antibody recognition (mutational analysis). Antibody cross-reactivity can be evaluated with arrays made from large numbers of unrelated synthetic peptides (3). In addition, peptide arrays can also be used to infer the substrate specificities of protein kinases (4–6) and to develop more potent peptide-based enzyme inhibitors (7). Peptide arrays are expected to play an increasingly important role in the development of new pharmaceutical targets and molecular medical diagnostics (1,7–11).

Two common methods for characterizing antibodies are enzyme-linked immunosorbent assay (ELISA) and synthetic peptide arrays on membrane support (SPOT synthesis) (12). Typically, in ELISA (13), dissolved peptides are aliquoted into microplate wells and allowed to passively bind to the polystyrene surface. These arrays can be made quickly and simply. However, it is not practical to array many different peptides in a quantitative ELISA because each peptide must be purified and its concentration measured accurately to ensure that each well contains the same amount of peptide. It is difficult to compare quantitative ELISA results obtained with different peptides because peptides are not likely to bind to polystyrene uniformly; wells that differ by peptide identity are also likely to differ by peptide amount. In SPOT synthesis, peptides are synthesized directly on cellulose membranes that have been derivatized to act as solid-phase synthesis supports, producing high-density arrays in a 384-well microplate format. After chemically treating the membranes to remove synthetic peptide sidechain protecting groups, these membranes are used directly in antibody binding assays. Because peptides are covalently attached to cellulose membranes, variable peptide binding does not have to be considered when evaluating assay results. SPOT synthesis instruments that produce two membranes in a 36-hour period are commercially available. The amount of peptide in each SPOT array element is about 25 μg, much more than is needed for assays. When a saturating amount of peptide is used, the assays become less sensitive in distinguishing variations. SPOT membranes must be stripped and reused to make the assays cost-effective. Peptide synthesis and arraying are linked in the SPOT approach, and the number of elements in the array is limited by the

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Vol. 37, No. 3 (2004)
Here we describe a new approach for producing peptide arrays that combines the desired features of both ELISA and SPOT synthesis (Figure 1). A synthetic peptide with an amino-terminal cysteine residue is first linked to the carboxyl terminus of a reactive carrier protein via a peptide bond by intein-mediated protein ligation (IPL; References 14–16).

**Figure 1.** The intein-mediated peptide array (IPA) strategy. (A) Diagram showing the production of carrier protein-synthetic peptide product by intein-mediated protein ligation (IPL). The carrier protein is fused to the N terminus of an intein, followed by a chitin binding domain (CBD). The carrier protein possessing an active C-terminal thioester is generated by incubation of chitin-bound fusion protein in the presence of 2-mercaptoethanesulfonic acid (MESNA). A synthetic peptide possessing a N-terminal cysteine is ligated to the C terminus of the carrier protein via a native peptide bond. (B) Flowchart for arraying synthetic peptides after IPL. The ligation products are serially diluted and arrayed onto a nitrocellulose membrane in a grid as shown and then reacted with an antibody.
The ligation products are then arrayed onto nitrocellulose membranes with a simple dot-blot apparatus. As in ELISA, the production of arrays is quick and simple, the number of peptides in the array can be adjusted as needed, peptide synthesis is kept separate from arraying, and small peptide amounts are arrayed so it is economical to use each array only once. Similar to the SPOT synthesis method, the peptide amount in each array feature is normalized by using a carrier protein when the array is produced. Labor-intensive peptide purification and accurate quantification are unnecessary. Here we demonstrate the benefits of this intein-mediated peptide array (IPA) strategy and provide two examples of antibody characterization based on this novel method.

**MATERIALS AND METHODS**

**Synthetic Peptides and Antibodies**

Peptide antigens corresponding to Tyr-98 to Ala-106 (CYPYDVP-DYA) of hemagglutinin (HA) protein, Glu-410 to Lys-419 (CEQKLISEEDL) of human p53 were synthesized with an N-terminal cysteine and high-performance liquid chromatography (HPLC)-purified by New England Biolabs (Beverly, MA, USA). A fluorescent peptide [CDPEK(fluorescein)DS, Flu-P1; New England Biolabs] was synthesized and purified by HPLC with fluorescein conjugated to the sidechain amino group of the lysine residue. The crude HA peptide (CAGAGYPYDV-DYA) and its derivatives (see Figure 4A) were synthesized by Cell Signaling Technology (Beverly, MA, USA). Two unpurified HA peptides, P7 and P7(-C), were mixed at various ratios in the ligation with carrier protein. Anti-sera were raised against HA, myc, and p53 peptides in rabbits (Covance Research Products, Denver, PA, USA). Polyclonal antibodies were purified by peptide-affinity chromatography according to a previously described protocol (17). Monoclonal antibodies against HA were from Zymed Laboratories (San Francisco, CA, USA) and Cell Signaling Technology. Monoclonal phospho-tyrosine antibody (P-Tyr-100) was obtained from Cell Signaling Technology.

**Thioester-Tagged Carrier Proteins**

Four carrier proteins were evaluated in this study: Hhal methylase (Hha, 39 kDa, from *Hemophilus hemolyticus* (18)); paranyson (27 kDa, from *Dirofilaria immitis* (19)); maltose binding protein (MBP, 43 kDa, from *Escherichia coli* (20)); and chitin binding domain (CBD, 7 kDa, from *Bacillus subtilis* (21)). These proteins were expressed as fusion proteins using vectors provided as part of the IMPACT™ Protein Expression System (New England Biolabs). The gene encoding for Hhal methylase originally cloned in the pTYB1 vector (18) was subcloned and fused to the Mth RIR1 intein in the pMRB vector. This vector contains an intein found in the *Methanobacterium thermoautotrophicum rir1* gene (22). The other three carrier proteins were fused to the Mxe GyrA intein, which is from the *Mycobacterium xenopi gyrA* gene (14,17,22). Carrier proteins with a cysteine-reactive carboxyl-terminal thioester group were produced by inducing intein-mediated cleavage with 50 mM 2-mercaptoethanesulfonic acid (MESNA) in 20 mM Tris-HCl, pH 8.5, 0.5 M NaCl at 4°C for 12–16 h, as previously described (14).

**Carrier Protein-Synthetic Peptide Ligations**

Synthetic peptides (500 μM) and carrier proteins (1 mg/mL or 20–40 μM) were ligated overnight at 4°C in 100 mM Tris, pH 8.5, 10 mM MESNA (14). Ligation efficiency was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of aliquots of unligated and ligated carrier protein. To simulate crude peptides at different levels of purity, HA peptide P7 (CAGAGYPYDVPA) was mixed with P7(-C), a synthetic peptide without an amino-terminal cysteine (AGAGYPYDVPA), at various ratios. For the experiment shown in Figure 2C, the ligated product samples were dialyzed against phosphate-buffered saline (PBS; 10 mM potassium phosphate, pH 7.2, 0.9% NaCl) using Slide-A-Lyzer® Mini Dialysis Units.

**RESULTS**

**Rationale for IPA**

This new method for producing
Figure 2. Dot-blot assays of carrier protein-synthetic peptide ligation products on nitrocellulose membrane (0.45 μm). (A) Protein blotting was performed with HhaI methylase (Hha, column 1), paramyosin (column 2), hemagglutinin (HA) peptide (column 3), myc peptide (column 4), 10× myc peptide (column 5), Hha methylase-myc peptide (column 6), and paramyosin-myc peptide (column 7). Serial 3-fold dilutions were made into phosphate-buffered saline (PBS) before the samples were blotted onto a nitrocellulose membrane that was reacted with a polyclonal antibody against myc peptide. (B) A nitrocellulose membrane (0.45 μm) was arrayed with Hha (column 1), paramyosin (column 2), HA peptide (column 3), 10× HA peptide (column 4), myc peptide (column 5), Hha-HA peptide (column 6), and paramyosin-HA peptide (column 7) and probed with a polyclonal antibody against HA peptide. (C) Analysis of the effect of peptide purity. HhaI methylase was ligated to a mixture of two synthetic HA peptides, P7 (CAGAGYPYDPAYA) and P7(-C) (AGAGYPYDPAYA) at various ratios as indicated. The blot was probed with a monoclonal antibody against HA peptide. The ligation products and the peptides were 3-fold serially diluted and the amount of total peptide is indicated on the right side, except that in panel A, the amount of unligated myc peptide in column 5 (33.3 nmol) is 10-fold more than the amount in column 4, and in panel B, column 4 (33.3 nmol) contains 10-fold more HA peptide than column 3. In panels A and B, columns 1 and 2 contain only carrier protein (0.02 mM).
peptide arrays is based on the assumption that an intein-generated protein can serve as a carrier to enhance the binding of peptides to a membrane and the signal-to-noise ratio. The peptides are linked to the carrier by the use of IPL (14,15), also called expressed protein ligation (16). An intein is an internal sequence that is auto-excised from a protein precursor. This unusual posttranslational modification is the basis for an intein-mediated protein purification system (18). The protein of interest is expressed as an intein fusion protein that can be affinity purified and released from the affinity resin by inducing the intein cleavage with MESNA, resulting in a stable carboxyl-terminal thioester group (Figure 1A) (22). We hypothesize that this cysteine-reactive protein can be utilized as a carrier protein for arraying synthetic peptides (Figure 1B). The peptides are synthesized with capping chemistry so that only full-length peptides, but not incomplete peptide synthesis by-products, contain an amino-terminal cysteine. The peptides containing N-terminal cysteine are selectively ligated to the carrier protein carboxyl-terminus through a peptide bond. Ligation is conducted with a 12.5- to 25-fold molar excess of synthetic peptide, so synthetic peptide purity does not significantly influence the yield of the final carrier protein-synthetic peptide ligation product. Internal or a C-terminal cysteine in the peptide could affect the ligation efficiency for the N-terminal cysteine. The inclusion of 10 mM MESNA in the ligation reaction can reduce the effect of disulfide bond formation of cysteine-containing peptides. In addition, an internal or C-terminal cysteine may undergo transthioesterification with the carrier protein, but this side reaction will readily reverse due to the presence of MESNA. Unlike conventional chemical methods for conjugating synthetic peptides to carrier proteins, the stoichiometry of an IPL reaction is precisely one-to-one; therefore, the amount of peptide in each array feature is determined by the ligation capacity of the carrier protein instead of the absolute peptide amount or purity. When needed, the ligation efficiency can be readily evaluated by a mobility shift by SDS-PAGE.

Use of Ligated Carrier Proteins-
Peptide in Dot-Blot Assays

HhaI methylase and paramyosin were prepared as cysteine-reactive carrier proteins with carboxyl-terminal thioester groups from intein fusion constructs by inducing intein cleavage with 50 mM MESNA. Each carrier protein was then ligated separately to two synthetic peptides with amino-terminal cysteine residues, human c-myc (myc) and HA, and the ligation products were arrayed onto 0.45 μm nitrocellulose membranes. To evaluate the effect of ligation, unligated synthetic peptide was arrayed at equal or 10-fold the amount of peptide present in the ligation product (10-fold peptide samples were present in column 5 for myc peptide in Figure 2A and column 4 for HA peptide in Figure 2B). The dot blots were probed with polyclonal antibodies against myc peptide and HA peptide. The data showed that a higher sensitivity of detection was achieved if the peptides were ligated to a carrier protein (Figure 2). The magnitude of this effect was peptide-dependent; ligation had more of an effect for myc peptide (Figure 3).

![Figure 3. Dot-blot assays with different carrier proteins. (A) Hemagglutinin (HA) peptide was ligated to HhaI methylase (Hha, column 6), maltose binding protein (MBP, column 7), paramyosin (column 8), and chitin binding domain (CBD, column 9). Carrier proteins were used as controls (Hha, column 1; MBP, column 2; paramyosin, column 3; CBD, column 4) along with unligated peptide (column 5). The nitrocellulose membrane (0.45 μm) was probed with polyclonal antibody against HA peptide. (B) The effect of sodium dodecyl sulfate (SDS) or organic solvent on the binding and immunoreactivity of various carrier protein-μc products. The myc peptide was ligated to HhaI methylase (columns 1, 5, and 9), MBP (columns 2, 6, 10), or paramyosin (columns 3, 7, and 11). The untreated ligation products (columns 1–3) and myc peptide (column 4) in phosphate-buffered saline (PBS) were arrayed as controls. The ligation products along with the myc peptide (columns 8 and 12) were treated with 2% SDS (columns 5–8) or an organic solvent (20% methanol) (columns 9–12). Serial 3-fold dilutions were made into PBS containing the denaturant (SDS or methanol) before the samples were blotted onto a nitrocellulose membrane. Immunoblotting was performed with polyclonal antibody against myc peptide. (C and D) Binding and recovery of a fluorescent peptide (C) before and (D) after a mock blotting assay. The fluorescent peptide Flu-P1 was ligated to Hha (columns 1 and 2). MBP (columns 3 and 4), paramyosin (columns 5 and 6), and CBD (columns 7 and 8). The ligation samples and unligated Flu-P1 peptide (columns 9 and 10) were arrayed onto a nitrocellulose membrane (0.2 μm) using a dot-blot apparatus. (E) Each well was washed thrice with PBS, and the membrane was visualized under ultraviolet (UV) light. (D) After the mock blotting assay, the membrane was visualized under UV light. The samples in columns 1, 3, 5, 7, and 9 were dialyzed against PBS before arraying. The amount of peptide is indicated on the right side.](image-url)
2A) than for HA peptide (Figure 2B). In addition, both the HA and myc peptides exhibited higher immunoreactivity when ligated to HhaI methylase and less immunoreactivity when ligated to paramyosin. The influence by the carrier protein could be due to the better binding of HhaI methylase compared to paramyosin. The intensities observed in this dilution assay showed that 33,300 pmol of unligated myc peptide were approximately comparable to 1.5 pmol of myc peptide ligated to HhaI methylase (Figure 2A), corresponding to a

Figure 4. Dot-blot alanine-scanning assays to map the epitopes of two hemagglutinin (HA) antibodies. An alanine-scanning HA peptide library (as shown in A) was ligated to HhaI methylase carrier protein. The ligation products and peptides were diluted and arrayed onto two 0.45 μm nitrocellulose membranes (3-fold serially diluted in rows 1–4), along with unligated synthetic peptides (3-fold serially diluted in rows 5–8). One membrane was probed with (B) a monoclonal antibody against HA from Zymed Laboratories, and the other membrane was probed with (C) an anti-HA monoclonal antibody from Cell Signaling Technology.
Table 1. Peptides for Dot-Blot Analysis

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Protein Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>hRad 17 (Ser645)-P</td>
<td>CASELPAsQPQPFSA</td>
</tr>
<tr>
<td>S2</td>
<td>BAD(Ser128)-P</td>
<td>CGMEEELsPFGRGS</td>
</tr>
<tr>
<td>S3</td>
<td>PLD1(Ser561)-P</td>
<td>CRKFSKFsLYKQLH</td>
</tr>
<tr>
<td>S4</td>
<td>PFK-2(Ser466)-P</td>
<td>CVRMRRNasFTPPLS</td>
</tr>
<tr>
<td>S5</td>
<td>RYR2(Ser208)-P</td>
<td>CNRTRIsQTOSQV</td>
</tr>
<tr>
<td>T1</td>
<td>GCN2(Thr745)-P</td>
<td>CSDPSGHILGMVGTA</td>
</tr>
<tr>
<td>T2</td>
<td>PERK(Thr980)-P</td>
<td>CAYATHIhGQVTKL</td>
</tr>
<tr>
<td>T3</td>
<td>IRAK(Thr100)-P</td>
<td>CRARDIItAWHPPA</td>
</tr>
<tr>
<td>T4</td>
<td>DNA-PK(Thr2609)-P</td>
<td>CTPMFVhEiQASQGT</td>
</tr>
<tr>
<td>T5</td>
<td>B-Raf(Thr439)-P</td>
<td>CDRNRMKILGRRD</td>
</tr>
<tr>
<td>Y1</td>
<td>FcgammaRIIb(Thr292)-P</td>
<td>CGAEhBTItSLhMHP</td>
</tr>
<tr>
<td>Y2</td>
<td>CSF1R(Thr723)-P</td>
<td>CSQGVDTyVEMRPV</td>
</tr>
<tr>
<td>Y3</td>
<td>PKCdelta(Tyr64)-P</td>
<td>CFDAhlyhGERVhQ</td>
</tr>
<tr>
<td>Y4</td>
<td>PTEB(Tyr240)-P</td>
<td>CREDhFMFhEFhPOQ</td>
</tr>
<tr>
<td>Y5</td>
<td>C-Kit(Tyr719)-P</td>
<td>CSDSTNEyhMDMhKG</td>
</tr>
</tbody>
</table>

Four groups of peptides containing phospho-serine (S1–S5 as indicated in the column on the left side), phospho-threonine (T1–T5), phospho-tyrosine (Y1–Y5), or no phosphorylation (U1–U5) were ligated to thioester-tagged HhaI methylase as described in the Materials and Methods section. The names of the proteins from which the peptides were derived are shown in the middle column. The phosphorylated residues are indicated in lowercase in the peptide sequences (column on the right side).

22,000-fold increase in detection after ligation. In this experiment, the smallest effect of ligation was 27-fold, observed for HA peptide ligated to paramyosin. Compared to ligation products, excess unligated peptide in ligation reactions makes a negligible contribution to assay results.

Unligated HA peptide was bound more efficiently to nitrocellulose than unligated myc peptide. When making arrays with other peptides, we occasionally found other unligated peptides that were bound to membranes at levels as high as HA peptide (data not shown). The variable binding of unligated peptides may affect the accuracy of antibody binding assays in either dot-blot or ELISA format. The inability to accurately control the amount of unligated peptide bound to an assay surface can affect quantitative antibody binding assays in which more than one peptide is used. Furthermore, intensity differences between HhaI methylase-ligated myc peptide (Figure 2A, column 6) and HhaI methylase-ligated HA peptide (Figure 2B, column 6) are probably due to the titers or affinities of the antibodies against myc and HA as well as, to a smaller extent, the different binding affinities of the ligated products for the membrane. Similarly, results were obtained with 0.45 μm nitrocellulose, 0.2 μm nitrocellulose, and 0.2 μm nylon membrane, and with other peptide-antibody pairs, such as p53 (data not shown).

To investigate the effect of peptide purity on ligation efficiency and assay sensitivity, crude synthetic peptide with an amino-terminal cysteine residue was mixed with crude synthetic peptide without amino-terminal cysteine at various ratios (Figure 2C). The peptide mixtures were ligated to thioester-tagged HhaI methylase under standard conditions, with a 12.5-fold molar excess of peptide to carrier protein. Gel-shift assays showed approximately 10% of the carrier protein was ligated to synthetic peptide when the cysteine-containing synthetic peptide purity was artificially reduced to 30% (data not shown). However, this ligation product gave essentially the same immunoblot result as pure cysteine-containing synthetic peptide. When cysteine-containing peptide was further reduced to 10%, ligation to carrier protein was barely detectable, and immunoreactivity was significantly reduced. This demonstrates that unpurified synthetic peptides can be used in ligation reactions as long as their level of purity is at 30%.

Screening for Optimal Carrier Protein

Carrier proteins are ligated and arrayed as nonadenated, native proteins. The carrier protein chosen for ligation could affect peptide immunoreactivity and antibody binding. To evaluate this possibility, several cysteine-reactive carrier proteins, HhaI methylase, MBP, paramyosin, and CBD were generated. These proteins were selected because they have few or no cross-reactive epitopes with rabbit or mice sera, thus reducing nonspecific background in immunoblot assays, and the reactive thioester can be readily generated by the intein-mediated protein purification system.

HA peptide was ligated to the four carrier proteins, and the ligation products were arrayed on a 0.45-μm nitrocellulose membrane for a dilution dot-blot assay (Figure 3A). The results demonstrated that the HA peptide was most immunoreactive when ligated to HhaI methylase and least immunoreactive when ligated to CBD. With CBD as the carrier protein, the signal was almost undetectable, perhaps because CBD has the lowest molecular mass (7 kDa) of the carrier proteins that were evaluated. Immunoreactivity was not a simple function of carrier protein molecular mass because, when ligated to HA, MBP (43 kDa) did not give as strong a response as HhaI methylase (39 kDa), even though they both have similar molecular mass. Consistent with the results shown in Figure 2, paramyosin-HA peptide was only slightly less immunoreactive than the HhaI methylase-HA peptide.

Protein blotting was also performed to assess the effect of SDS or an organic solvent on the binding and immunoreactivity of various carrier protein products (Figure 3B). The HhaI methylase-myc peptide exhibited strong immunoreactivity after denaturation with 2% SDS or 20% methanol. Two percent SDS had a greater inhibitory effect on the binding efficiency of the carrier proteins. In addition, the paramyosin-myc sample showed no reduction in immunoreactivity after the treatment with 20% methanol, whereas the signal from the MBP-myc product significantly decreased, indicating that its binding capability is adversely affected.
Binding of Ligated and Unligated Fluorescent Peptide

To directly evaluate the effect of using a carrier protein on binding and retention, a peptide derivatized at a lysine sidechain with fluorescein, CDPEK (fluorescein)DS (Flu-P1), was ligated to four carrier proteins (HhaI methylase, MBP, paramyosin, and CBD). The dialyzed and undialyzed ligation products were arrayed on a 0.2-μm nitrocellulose membrane, along with unligated fluorescent peptide (Figure 3C). This membrane was then taken through a standard blotting assay procedure, omitting primary and secondary antibodies and using peptide fluorescence to track binding and recovery (Figure 3D). The membrane was visualized under UV light before and after the blotting assay.

Unligated Flu-P1 peptide was bound inefficiently to the membrane during arraying (Figure 3C, columns 9 and 10), as observed for myc peptide and HA peptide (Figure 2). Furthermore, excess Flu-P1 peptide in ligation reactions appeared to be trapped by carrier protein after arraying but was then washed off the membrane during the assay procedure. Before the mock blotting assay (with the primary and secondary antibodies omitted), undialyzed ligation products had higher levels of fluorescence than dialyzed ligation products, but at the end of the assay, the levels of fluorescence were similar. For example, the undialyzed HhaI methylase-Flu-P1 ligation product (column 2) contains more fluorescent peptide than its dialyzed partner (column 1) before (Figure 3C), but not after (Figure 3D), the mock assay. Among the four carrier proteins, HhaI methylase ligation products gave the highest level of fluorescence after the mock assay. The washing steps of the mock assay did not significantly change the level of fluorescence observed with dialyzed HhaI methylase-Flu-P1, which is consistent with its tenacious binding. This direct assay for peptide binding and recovery suggests that the amount of ligation product bound to the membrane has more impact on assay intensities than how the ligation product presents the peptide to the antibody; that is, HhaI methylase ligated peptide gave a stronger assay response than the paramyosin ligation product, most likely because of its higher binding efficiency to the membrane.

Epitope Mapping by Alanine Scanning

With an alanine-scanning HA peptide library, the IPA method was used to generate arrays to map and differentiate the epitopes of two commercially available monoclonal antibodies against HA. The HA peptide library consisted of a CAGAG peptide tag at the amino terminus of residues 98–106 of HA protein (YPYDVPDYA). The amino-terminal peptide tag allowed ligation to carrier proteins and served as a spacer for antibody binding. The library consisted of nine components, P1 through P9, one for each of the eight HA peptide residues substituted with Ala and with the P9 component corresponding to the unaltered HA peptide sequence (Figure 4A). These synthetic peptides were ligated to HhaI methylase carrier protein, and the ligation products were arrayed in duplicate on 0.45 μm nitrocellulose membranes along with unligated synthetic peptides. Each membrane was then probed with monoclonal antibodies against HA from either Zymed Laboratories (Figure 4B) or Cell Signaling Technology (Figure 4C).

This assay showed similarities and differences between the two HA monoclonal antibodies. Both antibodies tolerated alanine substitutions at residues 7 and 8. Alanine residues at positions 1, 2, and 4 reduced binding to nearly undetectable levels, indicating those positions are necessary parts of the epitope for both antibodies. However, the two antibodies recognized slightly different epitopes; the antibody from Zymed Laboratories was less sensitive to replacement at residues 5 and 6 than the antibody from Cell Signaling Technol-
ogy and, conversely, the Cell Signaling Technology antibody tolerated alanine at position, 3 more than the Zymed Laboratories antibody. Importantly, the unligated synthetic peptides showed no detectable signal, precluding this kind of detailed epitope analysis.

**Determining the Specificity of Phospho-Specific Antibody Using IPA**

We constructed an array of unrelated synthetic peptides to demonstrate the specificity of a general phospho-tyrosine antibody. Four groups of peptides containing phospho-tyrosine, phospho-serine, phospho-threonine, or no phosphorylated residue were ligated to HhaI methylase (Table 1). The ligation products were arrayed onto nitrocellulose for a dilution dot-blot assay and incubated with a phospho-tyrosine antibody (P-Tyr-100) that recognizes peptides containing phospho-tyrosine regardless of the surrounding peptide sequence (Figure 5A). One of the five unligated phospho-tyrosine peptides (Y4) yielded significant immunoreactivity, although at a level 27-fold lower than the same peptide ligated to carrier protein (Figure 5B). This phospho-tyrosine antibody (P-Tyr-100) did recognize all five phospho-tyrosine peptides, but with different affinities. Peptides Y1 and Y4 produced strongest signals, followed by Y2 and Y3, and then by Y5. Different levels of binding were especially clear for ligation products that were diluted at least 27-fold [i.e., for array features that contain 123 pmol peptide or less (Figure 5B)].

**DISCUSSION**

With IPL and simple laboratory techniques, peptides, containing natural or modified amino acids, can be uniformly arrayed onto membranes. A similar carrier protein-peptide ligation system could be devised using peptides with a terminal biotin residue and avidin (23,24) or chemically conjugated to a solid matrix (8,25,26). However, one of the significant advantages of IPA is that the synthetic peptides containing N-terminal cysteine can be used as antigens, for conjugation with keyhole limpet hemocyanin (KLH), as well as in arrays and Western blot analysis. Because peptides are ligated to carrier proteins through covalent bonds, the ligation products are stable and can be stored at -20°C for arraying at a later date. Another advantage of IPA over SPOT is that an array with a dilution series can be easily produced for quantitative assays. Producing arrays by IPL technology involves essentially only one step more than arraying unligated peptides, and this one step significantly makes binding to the array surface more uniform. The dot-blot apparatus presented here limits the number of array features to 96. However, ligation products can be arrayed with higher-density devices, including microarray printers. In practice, the number of features is limited by the number of peptides available for arraying. With the dot-blot apparatus, we recommend using HhaI methylase as a carrier protein and arraying at least 5
pmol ligation product (123 pmol of peptide). SPOT membranes in 384-well microplate format typically have 20 nmol peptide in each feature, about 160-fold more than the amount we recommend for this new method. Under these conditions, 1 mg of peptide is ligated in a volume of 1 ml, which after dilution for arraying produces 2700 array features, each 2 mm in diameter. Higher-density arraying devices could produce smaller features and more arrays from the same amount of ligation reaction.

In summary, the application of the IPA technique for generating peptide arrays has been demonstrated to significantly improve the signal-to-noise ratio. The advantages of this approach include increased retention of peptides to various membranes, ease of use, and the ability to normalize peptides of different concentrations and purities. In addition, the carrier protein may function as a spacer for increased sensitivity in peptide-affinity ligand interaction. Consequently, the results are more sensitive, consistent, and reliable.

ACKNOWLEDGMENTS

We sincerely thank Drs. Thomas C. Evans, Jr, William Jack, Nicole M. Nichols, Christopher J. Noren, Eric J. Cantor, Larry A. McReynolds, Elisabeth A. Raleigh, Roby D. Polakiewicz, Michael J. Comb, and Yi Tan for the valuable discussions. We also thank New England Biolabs Organic Division and Cell Signaling Technology Peptide Laboratory for peptide synthesis and Dr. Donald G. Comb for his support.

COMPETING INTERESTS STATEMENT

J.R. is employed by Cell Signaling Technology, the manufacturer of several products discussed in this paper. L.S., I.G., J.R.M., and M.-Q.X. are employed by New England Biolabs, the manufacturers of two of the products discussed in this paper.

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Received 12 March 2004; accepted 25 May 2004.

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