Video Article

Identifying Protein-protein Interaction Sites Using Peptide Arrays

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Abstract

Protein-protein interactions mediate most of the processes in the living cell and control homeostasis of the organism. Impaired protein interactions may result in disease, making protein interactions important drug targets. It is thus highly important to understand these interactions at the molecular level. Protein interactions are studied using a variety of techniques ranging from cellular and biochemical assays to quantitative biophysical assays, and these may be performed either with full-length proteins, with protein domains or with peptides. Peptides serve as excellent tools to study protein interactions since peptides can be easily synthesized and allow the focusing on specific interaction sites. Peptide arrays enable the identification of the interaction sites between two proteins as well as screening for peptides that bind the target protein for therapeutic purposes. They also allow high throughput SAR studies. For identification of binding sites, a typical peptide array usually contains partly overlapping 10-20 residues peptides derived from the full sequences of one or more partner proteins of the desired target protein. Screening the array for binding the target protein reveals the binding peptides, corresponding to the binding sites in the partner proteins, in an easy and fast method using only small amount of protein.

In this article we describe a protocol for screening peptide arrays for mapping the interaction sites between a target protein and its partners. The peptide array is designed based on the sequences of the partner proteins taking into account their secondary structures. The arrays used in this protocol were Celluspots arrays prepared by INTAVIS Bioanalytical Instruments. The array is blocked to prevent unspecific binding and then incubated with the studied protein. Detection using an antibody reveals the binding peptides corresponding to the specific interaction sites between the proteins.

Video Link

The video component of this article can be found at https://www.jove.com/video/52097/

Introduction

Protein-protein interactions mediate most of the processes in the living cell. Impaired protein interactions may result in disease, making protein interactions important drug targets. It is thus highly important to understand these interactions at the molecular level. Protein interactions are studied using a variety of techniques ranging from cellular and biochemical assays to quantitative biophysical assays, and these may be performed either with full-length proteins, with protein domains or with peptides. Peptides serve as excellent tools to study protein interactions. This is because peptides can be easily synthesized and allow the focusing on a specific interaction site on one hand and on multiple protein targets in a high throughput manner on the other hand[1,2]. Peptide array screening is a fast, easy to perform method for obtaining a large amount of data about the interactions of a target protein with numerous partners in a short time[3]. Unlike other biochemical or biophysical methods for detecting and analyzing protein-protein interactions, peptide array screening requires a very low concentration of protein and can detect very weak binding. Peptide arrays can be used for many applications in peptide-protein interactions such as mapping of protein-protein or receptor-ligand interaction sites[4], homo- or hetero-oligomerization interfaces, characterizing antibodies epitopes[5], studying enzyme activities[6] and high throughput structure-activity relationship (SAR) studies[7]. For an in-depth review about peptide array screening see Katz *et al.* [4]

Several types of peptide arrays currently exist. There are two major synthetic strategies for making peptide arrays: synthesis of the peptides before attaching them to the solid support, or synthesis of peptides directly on the solid support, mainly using the SPOT technique[4,8]. The peptides are synthesized on the solid support usually by 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry[8]. Among the common synthetic schemes are peptide attachment through the N terminus (*e.g.,* JPT pepstar arrays[9]) and peptide attachment through the C terminus (*e.g.,* PEPSCAN pepchip arrays[10], JPT pepspot arrays[9] and INTAVIS celluspot arrays[11])[4]. The solid support can vary and so does the chemistry of the peptide coupling to it. Cys-terminated peptides can be attached to glass slides via the thiol group[12]. The N terminus of a peptide can be covalently bound to a hydroxyl group on the cellulose membrane through esterification of the amino acid attached[8].

Here we present a detailed protocol for screening peptide arrays as a method for studying protein-protein interactions. The array we used is the Celluspots array, which is a micro-array containing large amount of spots (duplicates of up to 384 spots) on a small cellulose membrane supported by a glass slide. This enables working with low volumes of protein and antibodies and obtaining significant amount of data per single experiment. This array also contains high peptide density that allows detection of low affinity binding. The array was used for mapping the STIL-CHFR interaction, which is highly important for controlling normal cell proliferation[13]. Uncontrolled interaction between the two proteins can lead

to the development of cancer. By mapping this interaction we found the specific binding site and binding residues[14]. This paves the way for designing rational inhibitors that inhibit this protein-protein interaction.

Protocol

1. Designing a Peptide Array

1. Divide the sequence of the target protein into partly overlapping 10-20 residues peptides. Vary the amount of overlap on the specific experiment and the resources of the performing lab, but in principle the longer the overlap the better. When designing the peptides take into account known secondary structure elements in the protein that can be responsible for the interaction.
2. Order the designed peptide array through commercial vendors. Here, use peptides covalently bound to the array through the C-terminus.

2. Blocking Non-specific Binding

1. Make a 50 mM Tris or Phosphate buffer solution containing 0.05% Tween 20 (TBST/PBST) and adjust to the desired pH with a measured amount of HCl or NaOH (in order to know the precise ionic strength) and the desired ionic strength with NaCl. Here, use a pH of 7.5 and an ionic strength of 150 mM.
2. Make a blocking solution of 2.5% (w/v) skimmed milk powder in TBST/PBST.
3. To prevent non-specific binding, immerse the array in 5 ml of blocking solution. Incubate the array for 2-4 hr at room temperature or overnight at 4 ºC on a shaker.

3. Incubating with the Protein

1. Wash the array first with 5 ml of blocking solution for 30 sec and then twice with 5 ml TBST/PBST for 5 min on a shaker at room temperature.
2. Incubate the washed array with 5 ml of His-tagged protein solution containing 2.5% (w/v) skimmed milk powder to prevent non-specific binding. Here, use 4.5 µM of protein solution (STIL 500-650) dissolved in the described blocking solution.
   NOTE: Usually 5-10 µM protein are used for the screening, but the protein concentration can be even as low as 2-3 µM, depending on the binding affinity and the local concentrations of the peptides that bound on the array (efficiency of the synthesis). The buffer in which the protein is dissolved can vary but is common to dilute the protein using the same blocking solution described above.
3. Incubate the array in the protein solution for 3-8 hr at room temperature or overnight at 4 ºC.

4. Incubating with the Antibody

1. Wash the array three times with 5 ml TBST/PBST. The first wash is for 30 sec followed by two 5 min washes on shaker at room temperature.
2. Incubate the washed array with 5 ml of diluted HRP-conjugated antibody (1:1,500) in an incubation buffer that contains the same ingredients at the same concentrations as the blocking solution, for 1 hr on a shaker at room temperature. The antibody can bind either the target protein or the tag.
3. Perform control experiments testing the interaction of the antibody with peptides on the array, especially if the array contains peptides from the target protein (*e.g.*, for oligomerization studies) by repeating the same protocol without step 3, incubating with the protein.
4. Wash the array three times with 5 ml TBST/PBST. The first wash is for 30 sec followed by two 5 min washes on shaker at room temperature.

5. Reading the Array

1. Carry out chemiluminescence development with an ECL western blotting substrate kit.
2. Perform the detection with a luminescent image analyzer.
3. Analyze the results. Make sure that both duplicates on the array show the same signals, so the results are reliable. If the structure of the protein partner is known, search on the structure for the binding peptides and look for the binding sites. Even peptides that are far in the sequence can be close together in the tertiary structure and create a binding site.

Representative Results

STIL is a highly important centrosomal protein. It controls normal cell division and cell proliferation[13,15–19]. STIL interacts with several proteins [18,20,21], and most of the interactions occur through its central part, which is an intrinsically disordered region (IDR)[14]. We designed an array composed of peptides derived from the STIL binding protein CHFR. CHFR is a tumor suppressor that is induced in response to mitotic stress[22]. Since the structure of the STIL binding domain of CHFR was unknown at the time, we divided the protein domain to 15 residues long peptides with overlap of 7 residues, as described in step 1.1 in the protocol. We performed a peptide array screening for identifying the CHFR-STIL binding sites, as detailed in the above protocol and illustrated in **Figure 1**. We used an INTAVIS Celluspot array composed of 384 peptides in duplicates, which can provide a huge amount of information as demonstrated in **Figure 2**. Using this peptide array we found the binding sites for STIL in its binding protein CHFR (**Figure 3**). Each black spot in the array represents a CHFR derived peptide that bound STIL. Note that each peptide appears twice since it exists in duplicate, which verifies the reliability of the results. The interaction between STIL and CHFR was previously demonstrated at the protein level[13,14]. The peptide array screening revealed 7 CHFR-derived peptides that bound STIL IDR, residues 500-650 (**Figure 3**, Amartely *et al.*[14]). These results allowed us to map the binding site of STIL on CHFR. Despite the distance of these 7 peptides on CHFR primary sequence they create a well-defined binding site for STIL in CHFR (**Figure 4**)[14].

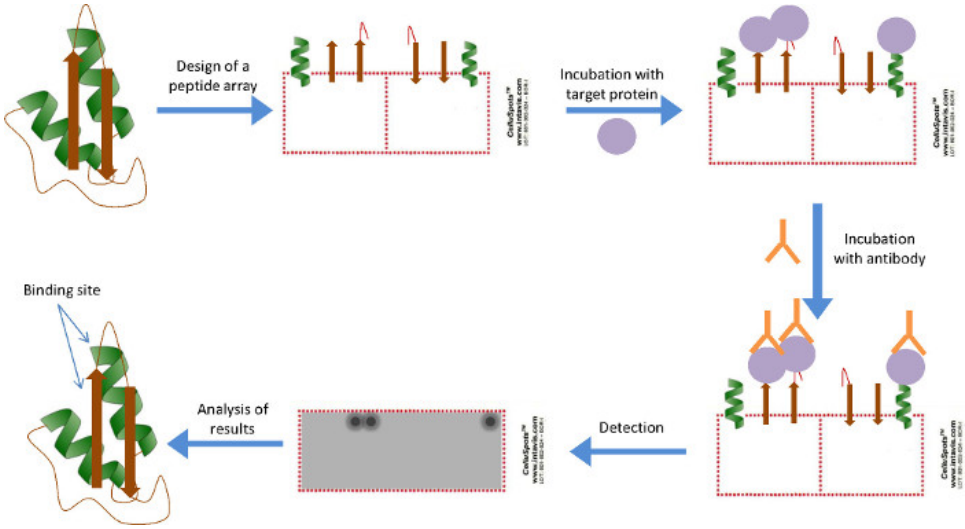

Figure 1: A scheme of peptide array screening. A- Designing a peptide array that consists of partly overlapping peptides derived from one or more partner proteins, taking into account their secondary structures; B- Incubating with the target protein; C- Incubating with an antibody for detection; D- Detection (can be done by chemiluminescence, fluorescence, *etc.*); E- Analyzing the results to reveal the protein binding sites. Please click here to view a larger version of this figure.

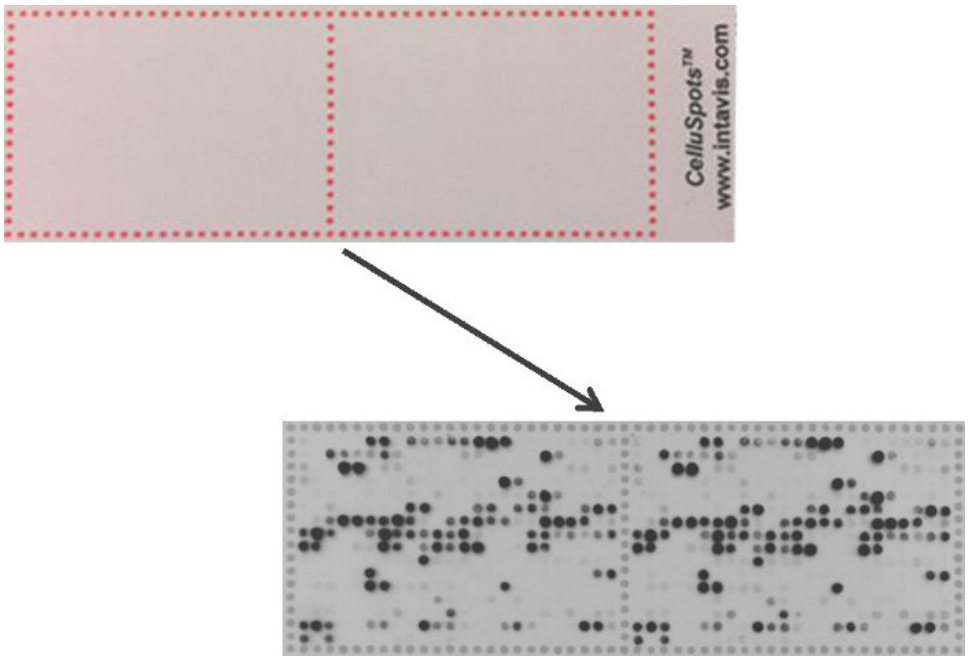

Figure 2: An example of peptide array screening. Detection achieved using chemiluminescence**.** The slide contains two identical arrays as duplicates. Please click here to view a larger version of this figure.

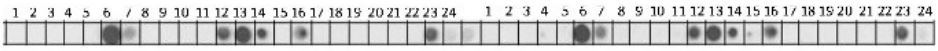

Figure 3: Screening a peptide array for binding of STIL IDR to CHFR protein: 4.5 µM His-tagged STIL 500-650 was screened for binding the array. Each black spot indicates binding between the STIL fragment and the CHFR-derived peptide[14]. Please click here to view a larger version of this figure.

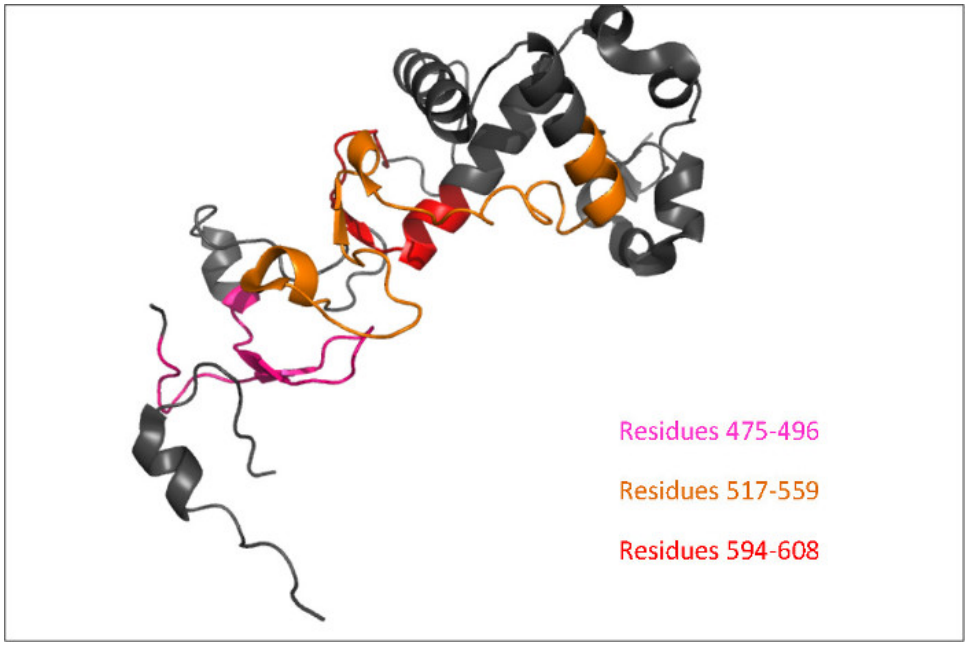

Figure 4:The STIL binding site in CHFR shown on the structure of the CHFR Cys rich domain (residues 424-661, PDB ID: 2XPO). The seven peptides that bound STIL in the peptide array screening can be divided to three regions colored pink, orange and red, which are distant in the primary sequence but close together in the 3D structure creating one binding site. This full region forms a binding pocket for STIL in the dimer CHFR[14]. Please click here to view a larger version of this figure.

Discussion

Peptide array screening is an excellent tool for identifying the binding sites of a target protein in its partners. This assay is fast and easy to perform and the results can be obtained in one or two working days. Peptide array screening is versatile and can be used for many purposes. It can be used for characterizing post translation modifications such as phosphorylation and identifying substrates of kinases. For example: the FLT3 kinase, which is related to acute myeloid leukemia, phosphorylates Tyr-containing substrate peptides found by a peptide array screening[6]. The inhibitory properties of the tyrosine kinase inhibitors pazopanib and lapatinib was tested using peptide array screening[23]. Peptides array can be used to identify the sites that mediate protein-protein interactions. Examples of such proteins studied in our lab using this method are: the interaction between the HIV-1 Vif protein and cellular A3G protein was characterized using a peptide array[24]; peptide array screening was used to study the interactions of the anti-apoptotic BCL-2 family with the pro-apoptotic ASPP2 protein[25] and to study the binding of tBID, a member of BCL-2 family, with the mitochondrial protein MTCH2[26]; the intra-molecular interaction between Myosin IIC domains was analyzed using peptide array screening[27]. Here we present an example of how we used peptide array screening for identifying the sites that mediate the STIL-CHFR interaction, a protein-protein interaction important in proliferation and cancer[14]. Revealing the exact binding site can serve as basis for the rational design of inhibitors of the studied interaction.

The peptide array screening is not a quantitative assay. It is semi-quantitative since the amount of peptide in each spot on the array varies due to difference in the synthesis yield and peptide purity. This in turn depends on the peptide sequence, length, *etc.* The variation in yield and purity can also lead sometimes to false negative or false positive results. Comparison between the intensities within the same array can be made, resulting in semi-quantitative ranking. However such comparison between different arrays or different experiments with the same array is not reliable. To quantify the binding, the peptides found in the peptide array screening should be synthesized and tested for binding the target protein using biophysical methods such as fluorescence anisotropy, isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), *etc.* Such assays are not always sensitive to weak binding since they require a high concentration of the protein to detect such binding. The peptide array screening, on the other hand, is very sensitive to weak binding. Thus, some of the interactions observed in the array screening could not be observed or quantified using methods such as fluorescence anisotropy[14]. The fact that peptide array screening requires very low concentrations of protein combined with the high sensitivity of the assay make the peptide array a useful method for screening protein-peptide interactions in a broad range of affinities.

False negative results can also be due to the fact that linear peptides may not properly represent the 3D structure of the binding site in the protein. This is however not a problem with intrinsically disordered proteins, which are well represented by peptides. False positive results can also be caused by binding of the antibody to the array. These can be detected by performing a control experiment as described in the protocol section 4.3. False negative results can also be obtained if the protein epitope that is recognized by the antibody become unexposed upon binding to the peptide array. This problem is not common but can be solved by using a polyclonal antibody against the protein.

The peptide array screening can also be used for improving lead peptides, including Alanine scan and SAR studies[7]. Once the binding site of the target is known, an array based on its sequence can be designed with multiple modifications including replacement of each residue to Alanine (Ala scan) to identify the important residues for the interaction. In a similar way, SAR studies can be performed by changing different properties of the binding peptides. These include varying the length of the peptide, replacing residues by the corresponding D-amino acids and other modifications such as methylation, glycosylation and replacement by non-natural amino acids. Such modified peptides, all based on one target

sequence, can be synthesized on one array and screened for binding the target protein in one fast and easy experiment, providing a wealth of information for improving a potential inhibitor and understanding the molecular mechanism of the interaction.

The methodology for peptide array screening is highly flexible and changes in the protocol can be applied when required. Other blocking compounds beside milk, such as bovine serum albumin (BSA) or sucrose, can be used. If non-specific binding is observed, higher concentrations of milk/BSA/sucrose should be used. The protein can be incubated with the array in any desired buffer, but it is important to add to the buffer milk / BSA / sucrose or any other blocker that is used in the blocking solution, to prevent unspecific binding. The protein concentration should remain relatively low, and strong binding can be observed even with 3-5 µM protein. The volume of incubation in all steps (blocking, incubation with the protein or antibody and washing) should be enough to fully cover the array. Usually 4-5 ml are enough. A Secondary antibody can be used if the primary antibody is not detectable. In this case steps 6 and 7 should be repeated with dilution and incubation times that fit the specific secondary antibody. The detection procedure depends on the antibody. For horseradish peroxidase (HRP) conjugated antibodies, chemiluminescence is commonly used as described in this protocol. Different alternatives can be used for different antibodies, such as fluorescence or electro-chemiluminescence[4]. The peptide array described herein is for single use only and cannot be reused.

The protocol presented here demonstrates the wide usefulness of peptide arrays. Using a correctly designed array, binding sites between two proteins can be identified in detail. Once the binding site is well characterized it can be used as a drug target site for inhibiting the relevant protein-protein interaction. Based on the binding peptides found in the array screening, inhibitory small molecules can be designed and screened as drug leads.

Disclosures

The authors have nothing to disclose.

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