Chapter 20

Kinome Profiling Using Peptide Arrays in Eukaryotic Cells

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Summary

Over the last 10 years array and mass spectrometry technologies have enabled the determination of the transcriptome and proteome of biological and in particular eukaryotic systems. This information will likely be of significant value to our elucidation of the molecular mechanisms that govern eukaryotic physiology. However, an equally, if not more important goal, is to define those proteins that participate in signalling pathways that ultimately control cell fate. Enzymes that phosphorylate tyrosine, serine, and threonine residues on other proteins play a major role in signalling cascades that determine cell-cycle entry, and survival and differentiation fate in the tissues across the eukaryotic kingdoms. Knowing which signalling pathways are being used in these cells is of critical importance. Traditional genetic and biochemical approaches can certainly provide answers here, but for technical and practical reasons there is typically pursued one gene or pathway at a time. Thus, a more comprehensive approach is needed in order to reveal signalling pathways active in nucleated cells. Towards this end, kinome analysis techniques using peptide arrays have begun to be applied with substantial success in a variety of organisms from all major branches of eukaryotic life, generating descriptions of cellular signalling without a priori assumptions as to possibly effected pathways. The general procedure and analysis methods are very similar disregarding whether the primary source of the material is animal, plant, or fungal of nature and will be described in this chapter. These studies will help us better understand what signalling pathways are critical to controlling eukaryotic cell function.

Key words: Kinase, Arrays, PepChips, Activity, Cells, Tissues, Purified kinases.

1. Introduction

The predominance of phosphorylation as a regulator of cellular metabolism has enticed many researchers to develop strategies for making descriptions of cellular phosphorylation events (I). Classically, kinase activity and protein phosphorylation were studied using in-gel kinase assays or Western blot-based gel shift
techniques that exploit the size difference between the phosphorylated and unphosphorylated forms of proteins. These are, however, fairly cumbersome techniques and do not allow the study of large numbers of samples. The situation has improved with the advent of phosphospecific antibodies in the late nineties, which recognize the phosphorylated forms of proteins but not their unphosphorylated counterparts. Employing these antibodies, phosphorylation events can be detected using classical Western blotting but also in, for instance, ELISA formats to allow high throughput screening for kinase activity-modifying compounds (2), or in tissue arrays that enable histological analysis of protein phosphorylation in hundreds to thousands of relevant tissue samples simultaneously (3). The main drawback remains that only one type of phosphorylation is studied per experiment. Recently, employing multicolour FACS, Irish et al. characterized phosphoprotein responses to environmental cues in acute myeloid leukaemia at the single cell level (4). The advantage of this approach is that the individual variation of cells with respect to the amount of phosphoproteins is assessed and requires very little material. But even this most advanced FACS technology does not allow simultaneous assessment of, typically, more than ten antibodies at one time. This has prompted investigators to explore techniques for studying cellular phosphorylation with little a priori assumptions as to the phosphorylation events involved.

Among the advanced of these approaches is the one that is commercially offered by Kinexus, which using a multiblot system that relies on sodium dodecyl sulfate-polyacrylamide minigel electrophoresis and multilane immunoblters to permit the specific and quantitative detection of 45 or more protein kinases or other signal transduction proteins at once (5). When used to its full extent this technology produces almost complete descriptions of cellular phospho-protein networks, although it still remains fairly labour intensive. Alternatively a proteomic approach may be chosen, which typically consists of a separation of phosphoproteins by, for instance, 2-D gel electrophoresis or chromatography, followed by mass spectrometry. Steady progress is made in this area, and 4 years ago using strong cation exchange chromatography at low pH to enrich for tryptic phosphopeptides, a first large-scale proteomic profiling of phosphorylation sites from primary animal tissue has been performed (6). For protein spots that can be detected and unambiguously identified, these approaches provide a powerful way of monitoring the expression and regulation of potentially hundreds of proteins simultaneously, but in practice it is hampered by the fact that the positions of scarcely more than two dozen protein kinases are available on 2D proteomic maps. This reflects the fact that – like most signal transduction proteins – protein kinases are present at very minute levels in cells, and are often undetectable by the most sensitive protein stains, whereas
procedures based on the purification of phosphopeptides and determination of peptide structure by MALDI is time consuming. The major advantage of this approach is that it is completely unbiased, for instance, using microfluidic compact disk technology this approach identified two novel phosphorylation sites in the human mineralocorticoid receptor (6).

A disadvantage of all these approaches is that they are focused on the static determination of the relative concentration of phosphoproteins, but do not address the actual activity of various cellular signalling pathways. (A popular comparison is the dashboard of a car where the milage indicator gives information as to the distance travelled, but gives no information as to the velocity at which this is occurring. For obtaining the latter information one uses the speedometer.)

Adaptation of array technology for measuring enzymatic activity in a parallel fashion seems an obvious solution for the earlier mentioned problems, and progress in this direction has been made with the preparation of protein chips for the assessment of protein substrate interactions (7-9) and the generation of peptide chips for the appraisal of ligand–receptor interactions and enzymatic activities (10-13). Houseman and Mrksich (14) showed that peptide chips, prepared by the Diels–Alder-mediated immobilization of one kinase substrate for the non-receptor tyrosine kinase c-Src on a monolayer of alkanethiolates on gold, allows quantitative evaluation of kinase activity. In 2004 we showed that 32P-γ-ATP phosphorylation of arrays consisting of 192 peptides that are substrates for kinases and spotted on glass by cell lysates from human peripheral blood mononuclear cells allowed the simultaneous description of the temporal kinetics of a multitude of kinase activities following stimulation with lipopolysaccharide (15). The same design of peptide array is also highly useful for studying signal transduction in plants (16) Although these studies showed the pan-eukaryotic applicability of studying signal transduction with peptide arrays, it appeared, however, that the amount of substrates on this array was insufficient to allow truly comprehensive descriptions of cellular signal transduction.

This consideration prompted us to study the effectiveness of an array with substantially increased numbers of substrates, the kinase I PepChip. It harbours peptide substrates from species in all branches of eukaryotic life, and also from prokaryotes. Many mammalian-derived substrates and also substrates identified in e.g. bacteria, yeast, fungi, plants, drosophila, birds, and viruses are present. This set of substrates was selected from the Phosphobase resource (http://phospho.elm.eu.org) (17, 18) and a full list of the peptides and the proteins from which they are derived is listed on http://www.koskov.nl. Arrays were constructed by chemically synthesizing soluble peptides, which were covalently coupled to glass substrates as described for the smaller
arrays. Kinase I arrays consisted of 1,176 different peptides, spotted 2 times per carrier, to allow assessment of possible variability in substrate phosphorylation. The final physical dimensions of the array were $25 \times 75$ mm$^2$, each peptide spot having a diameter of approximately 250 $\mu$m, and peptide spots being 620 $\mu$m apart. This design was highly successful, generating profiles from a variety of organisms and tissues from all branches of eukaryotic life (19–23). One of the interesting results from these arrays is the observation that a common set of peptide substrates is phosphorylated by all eukaryotic organisms. Thus diversity seen within the eukaryotic kingdom with respect to the primary structure of kinases is not reflected on the substrate level (19).

Recently a new array design, the kinomics PepChip, was introduced. It is based on phosphoproteins from the human protein reference database that has information from rats, mice, and humans alone. This array features three sets of 1,024 nonapeptides for which a phosphospecific antibody is available for each peptide (24).

PepChips allow a comprehensive detection of the cellular metabolism in lysates. Up-regulation or down-regulation of a particular kinase activity may lead to a cascade of cellular events. These can be fit into specific cell signalling pathways or cellular functions and as such assign specific characteristics to certain cells.

2. Materials

1. PepChip slides (PepScan, the Netherlands, http://www.pep- scan.nl, see Note 1). The slides can be stored up to 3 months at 4°C in the supplied tube/box.
2. 60-mm Glass coverslips.
3. Lysis buffer for mammalian cells: M-PER® Mammalian Protein Extraction Reagent (Pierce) with dilution buffer: 1M stock of HEPES, pHi 7.5 (Sigma).
4. Plant cells lysis buffer: 20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM Na$_3$P$_2$O$_7$ (sodium pyrophosphate), 1 mM β-glycerophosphate, 1 mM Na$_2$VO$_4$ (sodium orthovanadate), 1 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotonin. Store at -20°C in aliquotes.
5. 100 mM PMSF (add fresh from stock in iso-propanol or acetone).
6. Additional stock reagents required: (all Sigma) 1 M MgCl$_2$; 1 M MnCl$_2$; 100 mM sodium orthovanadate; 1 M DTT; 100 mM ATP; 3% Brij-35; 20% PEG 8000; 100 mg/ml; 50% glycerol; 5 mg/ml BSA; 1 M NaCl; 1 M EDTA; 1 M EGTA;
3. Methods

3.1. PepChips Using Cells

All lysates and purified kinases should be kept on ice throughout.

1. Lyse 10⁶ cells in 100μl of M-PER lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF (add PMSF fresh before use).

2. From this take 33μl of lysate and add 67μl of 60 mM HEPES, pH 7.5, and centrifuge at 16,100 x g for 10 min at 4°C.

3. From this take 70μl and add 10μl of activation mix (activation mix = 70 mM MgCl₂ + 70 mM MnCl₂ + 400 μg/ml PEG 8000 + 400 μg/ml BSA).

4. Add 8μl of 50% glycerol.

5. Add 2μl of 32P-γ-ATP and centrifuge for 3 min at maximum speed. (To be carried out in the isotope lab with appropriate protection.)

6. Pipette out 90μl of this mix onto a glass coverslip and invert the PepScan slide onto the coverslip. Make sure to completely remove any trapped air bubbles (see also Note 7).

7. Incubate the PepScan slides in a heated water bath or stove at 37°C (in order to prevent drying out of the slides) for 90–120 min.

8. After the incubation, remove the coverslips off the slides by dipping them in PBS containing 1% Tween 20. Do this a few times till the coverslips completely slip off the glass slides.

9. Wash the slides twice in 2 M NaCl with 1% Tween 20, twice in PBS with 1% Tween 20, and then twice in demineralized water.

10. Airdry the slides.
11. Expose the slides to the phospho-imager plate and scan using a scanner.
12. Analyse the image using ScanAlyze (see Note 2).

3.2. PepChips Using Biopsies

1. Lyse the biopsy/tissue sample in lysis buffer in a final concentration of 0.4 mg biopsy/μl lysis buffer.
2. Centrifuge at 16,100 × g for 10 min at 4°C and discard the pellet.
3. Measure the protein, and the optimal concentrations for performing PepChips range between 70 and 100 μg/50 μl.
4. To 70 μl of the lysate, add 10 μl of the activation mix (activation mix = 70 mM MgCl₂ + 70 mM MnCl₂ + 400 μg/ml PEG 8000 + 400 μg/ml BSA).
5. Add 8 μl of 50% glycerol.
6. Add 2 μl of [γ-32P]ATP and centrifuge for 3 min at maximum speed. (To be carried out in the isotope lab with appropriate protection.)
7. Follow the same procedure as in step 6 from cell protocol.

3.3. PepChips Using Plant Tissue

1. Lyse 100 mg of leaf material in 200 μl of lysis buffer; add 1 mM PMSF fresh from stock.
2. Incubate on ice for 5 min.
3. Spin down 10 min at maximum speed (16,100 × g) at 4°C.
4. Add supernatants to a 0.2-μm filter and spin again 2 min at maximum 10,000 × g to remove particles.
5. Prepare activation mix fresh from stocks by mixing 0.33 ml glycerol (50%), 5 μl ATP (100 mM), 20 μl MgCl₂ (1 M), 3.3 μl Brij-35 (3%), and 10 μl BSA (5 mg/ml).
6. If necessary dilute the lysate in lysis buffer (this can remove background greying of the slides).
7. Add to 50 μl lysate 11.25 μl activation mix and 3 μl [γ-32P]ATP. (To be carried out in the isotope lab with appropriate protection.)
8. Pipette 60 μl on a coverslip and invert the PepScan slide onto the coverslip. Make sure to completely remove any trapped air bubbles.
9. Incubate the PepScan slides in a heated water bath at 30°C (in order to prevent drying out of the slides) for 2 h.
10. After the incubation, remove the coverslips off the slides by dipping them in PBS-containing 0.05% Tween 20. Do this a few times till the coverslips completely slip off the glass slides.
11. Wash the slides in PBS with 0.05% Tween 20, twice in 0.5 M NaCl and then twice in MilliQ water; perform all wash steps 5 min while shaking.
12. Airdry the slides.
13. Expose the slides 1 week to the phospho-imager plate and scan using a scanner.
14. Analyse the image using ScanAlyze.

3.4. PepChips Using
Purified Kinases

1. Take 70 µl of the desired protein kinase incubation mix (×ng or µg/ml of kinase catalytic subunit, 60 µM HEPES, pH 7.5, 3 mM MgCl₂, 3 mM MnCl₂, 3 mM Na₂VO₄, 1.2 mM DTT, 50 µM ATP, 0.03% Brij-35, 50 µg/ml PEG 8000, 50 µg/ml BSA).
2. Add 8 µl of 50% glycerol.
3. Add 2 µl of 32P g-ATP and centrifuge for 3 min at maximum speed. (To be carried out in the isotope lab with appropriate protection.)
4. Follow the same procedure as in step 6 from cell protocol.

3.5. Analysis

1. Use ScanAlyze software to analyse the PepChip images that were scanned. Before entering slides in the ScanAlyze software make sure to put the label down, convert to tiff format, invert, and put to 8 bits/channel. Table 1 shows an example of the PepChip properties that should be used for the analysis.
2. Use grid tools to determine spot size (usually 10 pixels) and position to obtain spot intensities and background intensities.
3. Import data from the individual experiments to an excel sheet for further analysis.
4. Use control spots on the array for validation of spot intensities between the different samples.
5. Exclude inconsistent data (i.e. SD between the different data points >1.96 of the mean value) from further analysis (see also Note 3).
6. Average spots and include for dissimilarity determination to extract kinases of which activity was either significantly

| Table 1 |
PepChip properties|

<table>
<thead>
<tr>
<th>PepChip</th>
<th>Spots per grid</th>
<th>Grids per replicate</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>6 (h) × 4 (v)</td>
<td>2 (h) × 4 (v)</td>
<td>2 (h)</td>
</tr>
<tr>
<td>Kinase 1</td>
<td>7 × 7</td>
<td>4 × 6</td>
<td>2 (v)</td>
</tr>
<tr>
<td>Kinome</td>
<td>8 × 8</td>
<td>4 × 4</td>
<td>3 (v)</td>
</tr>
</tbody>
</table>
induced or reduced. Various statistical tests are appropriate, for instance a heteroscedastic two-tailed Students' t-test (see also Note 5).

7. If two conditions are applied, statistically significant differences are used to generate provisional signal transduction schemes based on the available knowledge on signalling (see Note 6).

8. Alternatively, Venn diagrams can be constructed. In this case a cut-off criterion is defined (e.g. all spots having an intensity of more as 2 times the standard deviation of the background are considered positive) and shared and unique spots between the treatments are defined.

9. Purified kinases can be tested directly on the chip to define substrate specificities.

10. For establishing consensus phosphorylation sites, after in vitro phosphorylation of the array with a purified kinase, different peptides are incorporating wildly different amounts of $^{33}$P. It is possible that a peptide could be phosphorylated at more than one residue, which would mean that a peptide that, for instance, is phosphorylated at two serines adjacent to each other could result in a higher intensity than a peptide phosphorylated on one serine and this would mask that peptide which could have been left out of the analysis. Hence, only those peptides that had a single phosphorylation site should be considered, i.e. only those peptides that had a single serine, threonine, or tyrosine residue at the central position. (Of the 1,176 peptides, 354 peptides have a single serine, threonine, or tyrosine residue.)

11. Peptides are aligned manually relative to the centrally fixed serine, threonine, or tyrosine residue and ranked on the mean intensity of the replicas for each spot. For deriving the consensus sequence using arrays with 1,176 substrates, we consider only positions $-3$, $-2$, $-1$, $0$, $+1$, $+2$ ($+3$). For arrays with 1,024 spots we also take $-4$ and $+4$ into consideration, since the peptides on these arrays have a length of nine amino acids. Furthermore, we only select peptides with cut-off intensities within 50% of the peptide with the maximum intensity, and the relative contribution of each individual amino acid at each individual position is calculated and corrected for the relative abundance of that amino acid at that position relative to the central serine, threonine, or tyrosine.

3.6. Discussion

The phosphorylation of the arrays is usually robust and clear (see e.g. Figs. 1-3), but highly sensitive to particulate matter. The array is however as specific as the substrates are and thus validation remains important. Western blot is a possibility but measures the combined effect of kinases and phosphatases on a substrate,
Fig. 1. Representative images of PepChips. Kinase I PepChips as they appear after scanning of the phosphorimeter plate. Lysates used for these images were derived from cells of *Homo sapiens* (human, a) or *Arabidopsis thaliana* (plant, b). Chips were incubated for 2 h with the lysate and exposed on the phosphorimeter for 3 days.

Fig. 2. Representation of gridding in ScanAlyze. ScanAlyze enables you to adjust the grids as a whole, per set or even individual spots. Background noise can be flagged, in order to omit it in further analysis. This example depicts part of a kinase I slide, which contains two times 24 grids of seven times 7 spots.
Fig. 3. Correlation plot obtained after gridding showing the robustness of the experiment. Spot intensities of the technical replicate present on the kinase I chip are compared to obtain a measure of reproducibility (R).

and thus, at least theoretically, in vitro kinase assays are superior. Phosphoflow is a good alternative with respect to the limited number of cells needed. For tissues, phosphoimmunohistochemistry may be appropriate. If results are tricky, an α-ATP control (radioactivity not at the γ position but at the α position and thus inaccessible for a kinase reaction) should be considered. If this control is negative, results are very reliable.

Important future developments will include total validation of chips employing purified variants of all kinases from mammalian and possibly plant genomes, which should significantly enhance the value of this technology and help interpreting results. Also, the development of new software tools will greatly enhance the ease by which meaningful analyses can be generated. As these efforts at moment receive substantial support from the Dutch government, it is to be expected that the usefulness of peptide arraying will increase quickly. Together, these developments now quickly establish peptide arraying as the technology of choice for generating comprehensive description of eukaryote signal transduction.

4. Notes

1. More information on the PepChips can be obtained from http://www.pepscan.nl.
2. ScanAlyze can be downloaded free by academic users from the Eisenlab Web site: http://rana.lbl.gov/.
3. If two conditions show wildly divergent phosphorylation patterns (i.e. when two conditions are plotted on each other, most spots are on the X and Y axis), most likely a gridding error has been made (grid wrongly placed).

4. Radioactive α-ATP contains traces of radioactive γ-ATP (approximately 5%), and so low signals may be obtained when using this control.

5. On one slide the individual sets of spots, when plotted on each other should show an $R^2$ of at least 0.6. Otherwise the experiment is best repeated.

6. For completely sequenced organisms, Blast can reveal potential kinase targets. Blast results can be found on http://www.koskow.nl.

7. Slides should be absolutely horizontal during incubation; otherwise gradients appear even when the slide is fully covered with fluid.

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References


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