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Peptide microarrays for detailed, high-throughput substrate identification, kinetic characterization, and inhibition studies on protein kinase A

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ABSTRACT

A microarray-based mix-and-measure, nonradioactive multiplex method with real-time detection was used for substrate identification, assay development, assay optimisation, and kinetic characterization of protein kinase A (PKA). The peptide arrays included either up to 140 serine/threonine-containing peptides or a concentration series of a smaller number of peptides. In comparison with existing singleplex assays, data quality was high, variation in assay conditions and reagent consumption were reduced considerably, and assay development could be accelerated because phosphorylation kinetics were monitored simultaneously on 4, 12, or 96 arrays. PKA was shown to phosphorylate many peptides containing known PKA phosphorylation sites as well as some new substrates. The kinetic behavior of the enzyme and the mechanism of inhibition by AMP–PNP, staurosporin, and PKA inhibitor peptide on the peptide microarray correlated well with data from homogeneous assays. Using this multiplex setup, we showed that the kinetic parameters of PKA and the potency of PKA inhibitors can be affected by the sequence of the peptide substrate. The technology enables kinetic monitoring of kinase activity in a multiplex setting such as a cell or tissue lysate. Finally, this high-throughput method allows fast identification of peptide substrates for serine/threonine kinases that are still uncharacterized.

Protein kinases play an important role in cellular housekeeping. They translate signals from the outside world into cellular responses through a cascade of activation/deactivation reactions. These signal transduction pathways play a central role in events such as growth, proliferation, apoptosis, and ion fluxes. Deregulation of these signaling processes can cause uncontrolled cell proliferation. Therefore, understanding the properties, the regulation of activity, and the interactions of protein kinases is important for the design of compounds aimed at controlling unfavorable activities or interactions.

As a first step toward understanding the behavior of kinase inhibitors in a cellular context, and ultimately in vivo, the effect on the target kinase is usually studied in vitro. Using a single substrate peptide, concentrations of both ATP and peptide are varied, and the effect of inhibitors is studied. This does not reflect the intracellular situation in which interactions with multiple proteins determine the outcome of kinase action. Disturbance of some interactions, but not of others, may direct the kinase activity into a desired direction. When multiple peptide sequences are tested, the substrate preference of a kinase can be revealed, and this may give a lead to allow fine-tuning of inhibitor effects on its activity. Using standard kinase assays, such an approach is laborious and time-consuming, whereas the use of a peptide microarray makes such investigations feasible in a short time frame while using very small amounts of reagents such as recombinant expressed kinases. Many of the kinase assays used to date have not been optimized to substrate peptide sequence, and often the same peptide is used in assays for different kinases for practical reasons. These limitations are overcome by the multiplex kinase assays reported here. Many examples of the success of such a peptide microarray-based approach for substrate optimization have been shown. Already in 1995, Luo and coworkers [1] used an array-based method to identify and optimize substrates for protein kinases, including protein kinase A (PKA).[1] Comprehensive overviews of the methods used for the preparation and applications of peptide microarrays are found in Refs. [2,3].

The PKA catalytic subunit has become the archetypal protein kinase because it is the first one for which the crystal structure was solved [4] and because it has been the subject of numerous studies. Many of them have described the substrate specificity of this enzyme, its kinetic behavior, interactions, and function in cellular

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[1] *Abbreviations used:* PKA, protein kinase A; CCD, charge-coupled device; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; cAMP, cyclic AMP; EGTA, ethyleneglycoltetraacetic acid; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; AICc, Akaike's information criterion; ANOVA, analysis of variance.

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systems. To validate the PamChip peptide microarray technology in this research area, we studied the kinetics of PKA-catalyzed peptide phosphorylation in real time.

Most microarray technologies use a two-dimensional surface as a support for immobilization of detector molecules. Peptide arrays were used mainly for substrate identification and optimization and for the study of inhibitor effects. This manual and low-throughput technology, however, was still open for improvement. The approach presented here not only allows automated, and therefore fast and easy, identification of substrates for the numerous serine/threonine kinases that remain to be characterized but also provides a tool for the multiparallel detailed analysis of the kinetic mechanism of kinases for application of these enzymes in pharmaceutical research. PamChip arrays are based on a porous three-dimensional layer of aluminum oxide [5] containing channels that extend from top to bottom through the material. These channels increase the surface area 500-fold, allowing immobilization of large amounts of peptide, with local concentrations as high as 1 mM. The use of purified peptides gives high-quality reproducible in-spot concentrations and facilitates the production of large numbers of identical arrays. On each array, up to 400 different peptides can be covalently coupled to the activated material. Because the sample (20–40 μl/array) can be actively pumped up and down through the pores, limitations due to diffusion are overcome and reaction times are very short. The physical properties of the material (translucent when wetted) allow real-time detection of fluorescent signals by a charge-coupled device (CCD) camera. The unbound fluorescent detection molecules present in the reaction mix do not interfere with the signal in the spots because of the specific design of the array and the small focal depth of the optical system.

The microarray technology described here has been used, with spotted oligonucleotides, for gene expression analysis [6] and copy number detection [7]. Spotting of peptides allows monitoring of protein kinase activity of both purified kinases and kinases in a complex environment such as a cell lysate [8–10]. The protein kinase in solution phosphorylates its peptide substrate(s) on the array. The ATP concentration can be varied from sub-micromolar to physiologically relevant (millimolar) concentrations. Phosphorylated peptides are detected by an anti-phosphoserine/threonine or anti-phosphotyrosine antibody that is fluorescently labeled either directly or indirectly. Such an array allows simultaneous measurement of the phosphorylation of all peptides by one and the same kinase while eliminating any variation in composition of the reaction mixture.

To illustrate the feasibility of such an approach, we studied the behavior of the catalytic subunit of PKA in the presence and absence of various inhibitors on a microarray containing either 140 peptide sequences derived from human proteins or concentration series of multiple peptides. We compared the kinetic parameters of PKA as determined in this multiplex assay with literature data and extended the kinetic studies by investigating the effect of protein sequence on kinetic parameters and inhibitory potential.

Materials and methods

Materials and reagents

PKA (the full-length human catalytic subunit type alpha) and PKA inhibitor peptide (TYADFIASGRTGRRNAI-NH_2) were obtained from Millipore (Charlottesville, VA, USA). Phospho-(Ser/Thr) PKA substrate antibody (number 5045, 0.4 mg/ml) was obtained from Cell Signaling Technology (Danvers, MA, USA), and mouse anti-rabbit immunoglobulin G–fluorescein isothiocyanate (IgG–FITC, 0.4 mg/ml) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Abl kinase assay buffer and bovine serum albumin (BSA) were supplied by New England Biolabs (Ipswich, MA, USA), staurosporin was obtained from BioMol (Plymouth Meeting, PA, USA), and AMP–PNP was obtained from Roche Applied Science (Mannheim, Germany). Sypro Ruby Protein Blot Stain was obtained from Molecular Probes (Invitrogen, Eugene, OR, USA). All other chemicals used were of analytical grade.

The serine/threonine kinase PamChip microarrays (PamGene International BV, 's-Hertogenbosch, The Netherlands) comprised 140 serine/threonine-containing peptides. In addition, 4 phosphorylated peptides were present per array as positive controls, resulting in 144 peptides per array. Each peptide represents a 13-amino-acid sequence derived from a putative phosphorylation site in human proteins. UniProt Knowledgebase protein identities, amino acid position numbers, and protein descriptions were used for peptide naming (www.expasy.org/sprot). Via a spacer containing diethyleneglycol moieties and an N-terminal cysteine residue, the peptides are covalently immobilized on activated aluminum oxide [5] via thiol-reactive surface chemistry (maleimide function) at a spotting concentration of 1000 μM. A peptide may contain more than one phosphorylation site. The specific PKA arrays consisted of a number of peptides spotted in nine concentrations ranging from 50 to 1500 μM, including the CREB peptide (EILSRRPSYRKIL) derived from the cyclic AMP (cAMP) response element binding protein. The specific microarray used for antibody optimization contained a concentration series of phosphorylated CREB peptide (EILSRRPpSYRKIL). Some experiments were performed on arrays containing 14 different peptides, including 5 PKA substrates. Peptides were spotted in duplicate in six concentrations ranging from 100 to 1000 μM.

The concentration of immobilized peptides was checked by measuring the fluorescence (Cy3 filter) on at least four arrays after staining of the arrays with Sypro Ruby Protein Blot Stain for 15 min on a PamStation 4 instrument. Signal quantification was performed with Bionavigator software. Signal intensities were compared with a database of cumulative historic signal intensities for the arrays. Deviations from historic average values needed to be less than 10%. For concentration series, signals were linear with spotted peptide concentration.

Instrumentation

Incubations and kinetic reading of the PamChip peptide microarrays were performed on a PamStation 4 or PamStation 96 instrument (PamGene International BV), which allows fully automated incubation, washing, and imaging of 4 or 96 microarrays simultaneously. During incubation, the incubation mixture is pumped up and down through the array at a frequency of two times per minute. When the solution is underneath the array, fluorescent imaging of each array is performed. The instruments contain a 12-bit CCD camera for imaging. Appropriate filters allow imaging in different wavelength ranges suitable for FITC, Cy3, or Cy5 fluorescence. The focal depth of the camera is 40 μm, whereas the material has a thickness of 60 μm. During imaging, a wedge separates the bulk of the solution from the array, reducing interference of unbound fluorescent label. This instrumental design permits recording images during the incubation and so allows monitoring of the reaction in real time.

Bionavigator software for quantification of signal intensities was obtained from PamGene International BV. SigmaPlot 9.0 and the Enzyme Kinetics Module were obtained from Systat Software (Point Richmond, CA, USA), and Prism 4 software was obtained from GraphPad Software (San Diego, CA, USA).

Assay conditions

All incubations were performed at 30 °C. Just prior to use, the PamChip peptide microarrays were blocked with 0.2% (w/v) BSA