Profiling of Enzymatic Activities Using Peptide Arrays

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Abstract: Applications of peptide arrays in the field of enzyme profiling are described in detail. Focus is on assay principles for measuring activities of kinases, phosphatases or proteases and on substrate identification/optimization for kinases. Additionally, several examples for reliable identification of substrates for other enzymes like lysine methyl transferases and ATP-ribosyltransferases are given. Finally, use of high-density peptide microarrays for the simultaneous profiling of kinase activities in complex biological fluids like cell lysates or lysates of complete organisms will be analysed. All published examples of peptide arrays used for enzyme profiling are summarized comprehensively.

Keywords: Kinase, peptide microarray, phosphatase, protease, SPOT synthesis, substrate specificity.

1. INTRODUCTION

Phosphorylation of proteins by protein kinases plays an essential role in the regulation of cellular processes such as signal transduction, cell proliferation and viability, differentiation, apoptosis, and metabolism. Information about substrate proteins and peptides is necessary to integrate kinases into their biological networks. This can provide the basis for understanding molecular origins of diseases and for potentially developing tools for therapeutic intervention. The discovery of more than 500 members of these enzymes in the human genome stimulated a growing interest in protein kinases. Consequently, high throughput technologies for determining kinase substrates have become a prerequisite for elucidating the huge number of potential phosphorylation events triggered by these kinases. This demand can be perfectly matched by peptide (micro)arrays, which have proved to be powerful tools for the rapid delineation of molecular recognition events. In this review we describe the application of peptide arrays for enzyme profiling with focus on kinase, phosphatase and protease research.

2. CHEMISTRY OF PEPTIDE ARRAY PREPARATION

There are two main principles for the preparation of peptide arrays: *in situ* synthesis directly on the array surface or immobilization of pre-synthesized peptide derivatives. Normally, yields of peptides synthesized on surfaces are high and consistent over the entire support surface from one array region to another. In 1991 two different technologies for the *in situ* preparation of peptide arrays were published. Light-directed, spatially addressable parallel chemical synthesis [1] is a synthesis technology permitting extreme miniaturization of array formats, however it involves sophisticated and rather tedious synthesis cycles. A major problem is the novel set of chemistries. If these chemistries are not optimized final quality of the surface-bound peptides will give false positive (if an impurity is active) and/or false negative results (if the target peptide sequence was not synthesized). An interesting alternative to circumvent this limitation is the use of photo-generated acids in combination with standard Boc-chemistry.

Alternatively, the SPOT synthesis concept developed by Ronald Frank is the stepwise synthesis of peptides on planar supports, such as functionalized cellulose membranes, applying standard Fmoc-based peptide chemistry [2, 3]. SPOT synthesis is technically very simple and flexible and does not require any expensive laboratory automation or synthesis hardware. Nevertheless, the degree of miniaturization is significantly lower as compared to light-directed, spatially addressable parallel chemical synthesis. SPOT synthesis is very flexible and economic relative to other techniques and was transformed from a semi-automatic procedure [4] into a fully automated system. The basic principle involves the spatially addressed deposition of defined volumes of activated amino acid derivatives directly onto a planar surface such as functionalized cellulose, aminated polypropylene [5] or aminopropylsilylated glass slides [6]. The areas contacted by the droplets represent individual microreactors allowing the formation of a covalent bond between the amino acid derivative and the surface function. The resulting spot size is defined by the dispensed volume as well as the physical properties of the surface used. This SPOT synthesis has been reviewed extensively [7-9].

A very elegant form of spatially addressed compound deposition makes use of modified colour laser printers. The cartridges are filled with a solvent/amino acid derivative mixture resulting in an activated amino acid solution during the laser induced melting process [10-12]. Analogous ink-jet delivery of activated amino acids to appropriate functionalized surfaces, such as membranes, microscope slides [13] or spinning surfaces in a CD-format [14] for automated synthesis of peptides, have been developed by a number of companies, but is not yet commercially available.

When large numbers of peptide arrays with the same sequences are required, immobilization of pre-synthesized peptides is more economical than *in situ* synthesis. Immobilization is also the method of choice for long peptide sequences, which normally have to be purified to obtain high quality products. Chemoselective immobilization reactions are of particular interest in the preparation of peptide arrays because they allow control over both the orientation and the density of the attached peptides.

Different chemoselective reactions were used for peptide microarray preparation in connection with enzyme profiling experiments. An aldehyde function at the surface of glass slides in combination with amino-oxy-acetyl moieties in the peptides [15-19] or

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cysteinyl-residues [15, 20, 21] was used for the preparation of peptide microarrays on glass slides. The reaction between cysteine residues and surface bound maleimide groups was used for preparation of peptide microarrays for kinase [22-27] and protease profiling [28]. It could be demonstrated that native chemical ligation, introduced by Dawson *et al.* [29] is well-suited for effective attachment of kinase substrate peptides containing an N-terminal cysteine residue to thioester modified glass slides [30-32]. A more sophisticated reaction for oriented immobilization of peptide derivatives was introduced by Houseman *et al.* [33]. A Diels-Alder reaction between benzoquinone groups on self-assembled monolayers and cyclopentadiene-peptide conjugates led to efficient covalent attachment of kinase substrate peptides that were efficiently phosphorylated by c-Src kinase [33]. Formation of amide bonds by Staudinger ligation between azide-modified phosphopeptides and appropriately phosphin-displaying glass surfaces was used for preparation of phosphopeptide microarrays enabling profiling of protein tyrosine phosphatase activities [34.] Regioselective immobilization of poly(desoxythymidin)-modified kinase substrates onto differently coated glass slides was reported for PKA and c-Src [35]. Photocleavable acrylamide labelled cysteine-containing kinase substrates were incorporated into peptide-acylamide copolymer hydrogel surfaces and v-Abl- or c-Abl-mediated phosphorylation was detected by MALDI-TOF/TOF subsequent to laser induced cleavage at the ß-(2-Nitrophenyl)-ß-alanine residue [36]. There are several additional chemistries used for the chemoselective immobilization of peptides onto different surfaces (comprehensively reviewed in [7]) like formation of covalent bonds by reaction of salicylhydroxamic acids with 1,3-phenyldiboronic acid derivatives [37-39] or semicarbazides with aldehydes [40-44] but no applications for enzyme profiling have been described so far.

Insertion of a spacer between the peptide and the surface is an effective way to improve the efficiency of enzyme/substrate or antibody/peptide interactions on surfaces, as demonstrated with FLAG epitope peptides recognized by the monoclonal anti-FLAG M2 antibody [45]. Additionally, for protein tyrosine kinase $p60^{c\text{-}src}$ only incorporation of the long and hydrophilic 1-amino-4,7,10-trioxa-13-tridecanamine succinimic acid building block spacer allowed effective phosphorylation of the glass surface-bound peptides [15]. Similar linker structures were used to space apart Peptide Nucleic Acid (PNA) tags from potential protease substrates [46] or protein tyrosine kinase substrates [47]. Inamori *et al.* reported that insertion of a PEG spacer between chemoselective attachment point and the kinase substrate sequence improved phosphorylation efficiency by c-Src but not by PKA [48]. Moreover, insertion of hydrophilic dextran structures between the surface and the presented peptides was described as necessary for efficient kinase substrate interaction [49].

An interesting alternative to spacers is the use of proteins decorated with peptides. MacBeath and Schreiber used covalently attached bovine serum albumin as a spacer molecule to present kinases with p42MAPK, PKA and CKII peptide substrates covalently attached to amino acid side chains of the albumin protein [50]. Alternatively, substrate peptides fused genetically to the C-terminus of human leptin were immobilized onto aldehyde modified glass slides [51]. Another method is the decoration of streptavidin with biotinylated peptides or phosphopeptides [52]. Sun *et al.* were able to demonstrate that biotinylated phosphopeptides immobilized onto streptavidin-coated glass surfaces could be efficiently dephosphorylated by phosphatases or isomerized by Peptidyl-Prolyl-*cis/trans*-Isomerases [52]. Nevertheless, Kimura *et al.* could not find detectable phosphorylation of streptavidin-bound biotinylated PKA substrates by PKA but were able to demonstrate phosphorylation of the same substrates if regioselectively immobilized via polythymine-deoxyribonucleoside tag onto aminosilane- or polycarbodiimide-coated glass surfaces [35].

3. LIBRARY TYPES FOR ENZYME PROFILING

A considerable number of different library types have been used for enzyme profiling and substrate identification with peptide arrays. One can distinguish two general types of libraries: knowledge-based libraries comprising peptides with sequences that are derived from naturally occurring proteins, and libraries that are designed "*de novo*", *i.e.* either consisting of randomly generated single peptides or peptide mixtures based on combinatorial principles.

The knowledge-based library is of particular interest when the protein target for the enzyme is known. Identification of the actual phospho-acceptor residue or cleavage site is achieved by scans of overlapping peptides ("peptide scans") derived from the sequence of the substrate protein [19]. Alternatively, libraries of peptides covering the sequence around each potential phospho-acceptor residue or protease cleavage site have been used [53, 54]. The availability of high density peptide microarrays enabled the systematic extension of this approach in a "proteomics-like manner" addressing either groups of proteins comprising all human Peptidyl-Prolyl-*cis/trans*-Isomerases in form of 3250 overlapping peptides or covering the complete proteome of human cytomegalovirus (17181 overlapping peptides) immobilized onto one standard industry glass slide. Incubation with kinases and fluorescence scanning subsequent to treatment with phosphospecific dye yielded proteome-wide detection of phosphorylation sites [55]. In a similar approach sequences of experimentally identified phosphorylation sites were comprehensively evaluated on peptide arrays [16, 17, 25, 26, 56-67].

An extension of the knowledge-based libraries concept is the introduction of post-translational modifications, *e.g.* phosphorylation of serine/threonine or tyrosine residues, methylation of arginine/lysine residues and acetylation of lysine residues, within the substrate sequences. This more adequately mimics the natural environment in which phosphorylation occurs, allowing the detection of peptides that become substrates only after an initial priming modification event. Such modifications can be introduced on-chip enzymatically after chemical synthesis of the unmodified peptides [19] or chemically by either using modified building blocks during the course of peptide synthesis [19] or by "on-chip-chemistry" using chemoselective chemical reactions as demonstrated by selective acetylation of lysine side-chains in microarray bound peptides [68].

Another type of knowledge-based libraries allows the mapping of protein interactions involving two discontinuous components that are far apart in the primary polypeptide structure but form a composite phosphorylation/dephosphorylation site in the natively folded protein. Two separate peptides are synthesized independently by a double peptide synthesis method on a single spot allowing the detection of synergistic pairs of peptides for protein tyrosine phosphatase 1B and for serine/threonine kinase Erk2 [69].

For *de novo* detection of kinase substrates both combinatorial approaches and randomly generated libraries of single peptides proved to be useful. Combinatorial libraries have one or more defined amino acid positions and a number of randomized or degenerated positions [31, 49, 70-74]. Only one particular amino acid is introduced at the defined positions while a mixture of amino acids is introduced at the randomized positions, resulting in a sub-library of different sequences in each single spot. The number of individual sequences per spot depends on the number of randomized positions and the number of different building blocks used for these positions. Once the amino acids that are productive for phosphorylation by a given kinase have been identified at the defined positions the remaining randomized positions must be de-convoluted using follow-up libraries. Combinatorial libraries were successfully used with cellulose membranes as the solid support. However, representation of each single sequence of a peptide mixture is not guaran-

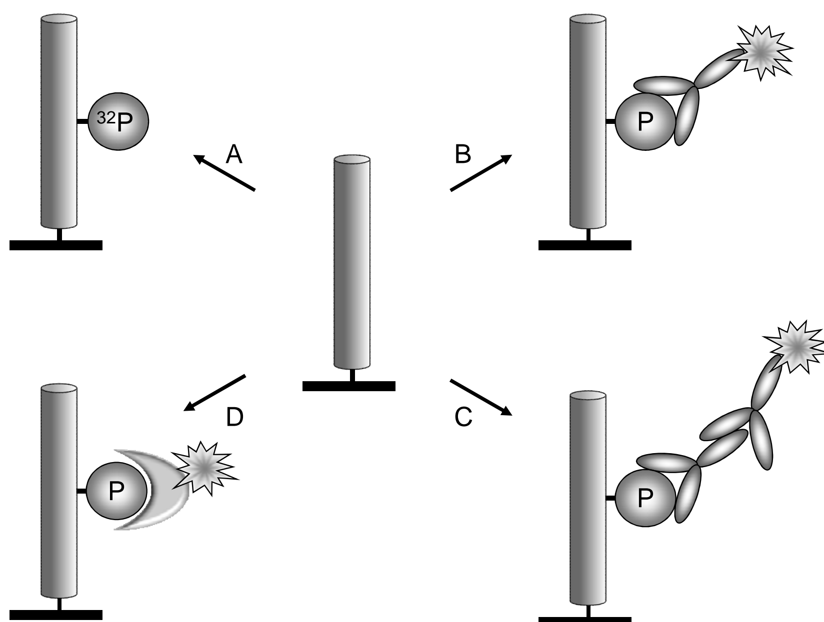

Fig. (1). Assay Principles for the Detection of Peptide Phosphorylation on Peptide Arrays. (**A**) The array is incubated with the kinase of interest in the presence of [γ-^{32}P or γ-^{33}P]-ATP and detection is performed by autoradiography. (**B**) Phosphorylation is measured with a directly fluorescently or chemoluminescently labelled anti-phosphoamino acid antibody. (**C**) Phosphorylation is measured with a generic anti-phosphoamino acid antibody in combination with a labelled secondary antibody. (**D**) Phospho-peptide detection using fluorescently labelled phospho-amino acid chelator.

teed when using peptide microarrays with a low concentration of peptide per spot on planar surfaces [31].

The tremendous miniaturization of peptide libraries possible on planar surfaces such as glass slides enables the application of randomly generated libraries of single peptides that cover a significant, although not complete part of the potential sequence space. Such randomly generated libraries for kinase substrate identification and kinase profiling have a defined phospho-acceptor residue and random sequences in the flanking areas [18]. In contrast to combinatorial libraries each spot represents one single sequence. If information on the consensus sequence for the substrates of a kinase is available random libraries can be biased by introducing defined positions derived from the consensus sequences.

Substrate characteristics, *i.e.* key interaction residues, can be deduced from all these library types using statistical analysis, provided that the number of identified substrates is high enough [17, 18, 71-75]. Alternatively, different library types based on single amino acid substitutions of identified substrate sequences, such as alanine scans or substitutional analyses [16, 58, 76, 77], permit comprehensive substrate characterization.

4. ASSAYS AND DETECTION

Measuring the activity of enzymes that modify peptides on microarrays involves either addition of chemical moieties to displayed peptides (kinases, acetyl transferases, glycosyltransferases, ADP-ribosyltransferases, etc.) or release of a part of the immobilized peptide derivative (proteases, phosphatases, demethylases, etc.). In principle, all enzymes modifying peptides or proteins can be applied to screen peptide arrays. However, so far only studies with kinases, phosphatases, proteases, lysine methyltransferases [78, 79], isomerases [80, 81], glycosyltransferases [82, 83], ADP ribosyltransferases [84], hydrolases [85], esterases [86] and SUMO ligases [87] have been described using peptide arrays or peptide microarrays.

We will review applications of peptide arrays for kinase, phosphatase and protease research in more detail.

There are 3 general assay principles applied to detect phosphorylated peptides on peptide arrays. One way is to incorporate a radioactive label during the phosphorylation reaction using [γ-$^{32/33}$P]-ATP (*see* Fig. **1A**). Subsequently, quantification of incorporated radioactivity is achieved using either a phosphorimager [17-19] or X-ray films, or alternatively photographic emulsions that deposit silver grains directly onto the glass surface [50]. This procedure has a low limit of detection and is only influenced by the selectivity of the kinase. Incubation protocols have been described for peptide arrays prepared by SPOT synthesis [88, 89] and peptide microarrays [17-19, 62, 90].

Alternatively, phospho-peptides can be detected using either phospho-specific antibodies (*see* Fig. **1B** and **1C**) or phosphospecific chelators (Fig. **1D**) that are labelled with a detection moiety. The detection moiety can be a fluorescent label such as fluoresceine [17, 20, 30] or an enzyme, for example horseradish peroxidase generating a chemiluminescent signal in combination with an appropriate substrate [69]. The detection moiety can be coupled either directly to the anti-phospho-amino acid antibody (*see* Fig. **1B**) [17, 31, 56] or to a secondary antibody (*see* Fig. **1C**) [33, 69]. Quality of anti-phospho–amino acid antibodies as detection tools was compared to the radioactive detection method generally recognized as the golden standard with respect to reliability. Here, the different detection procedures were applied to peptide microarrays on glass slides with 694 peptides derived from annotated phosphorylation sites from human proteins together with all their possible 2234 monophosphorylated derivatives [17]. Monoclonal anti-phospho-tyrosine antibodies only showed reliable results with no detectable binding to non-phosphorylated amino acids. Anti-phospho-serine antibodies, however, had an extremely high false-negative rate, while anti-phospho-threonine antibodies showed significant cross-reactivities for peptides with phospho-tyrosine. Similar results were described using peptide microarrays displaying more than 6000 phospho-tyrosine peptides derived from human phosphorylation sites in triplicates [68].

Additionally, phospho-amino acid chelators coupled to a detection moiety can be used (*see* Fig. **1D**). Martin *et al.* described the Pro-Q Diamond phospho-sensor dye which recognizes phosphopeptides with remarkably little cross-reactivity and a low false negative rate [91]. It was demonstrated that this dye could be ap-

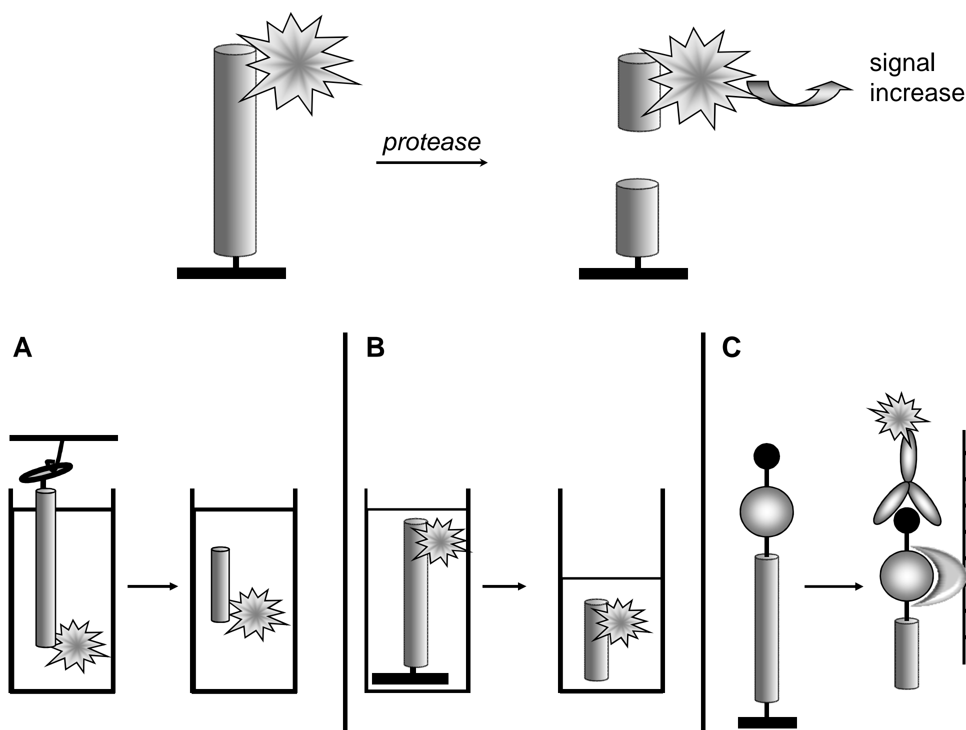

Fig. (2). Detection of Proteolytic Cleavage on Peptide Arrays Reading Released Fragment. (**A**) N-terminally labelled immobilized peptides in the 96-well plate format, cleavage yields fluorescently labelled fragment released into the well of the microtiter plate; membrane disk with bound peptide fragment is released before fluorescence measurement [111]. (**B**) Alternative assay with N-terminally labelled immobilized peptides in the 96-well plate format, membrane disk with bound peptide or peptide fragment will remain in the well of the microtiter plate but released fragment is separated from cellulose membrane by transfer of aliquots of supernatant to another microtiter plate which yields fluorescence signals for cleaved peptides [112-114, 147], and (**C**) indirect detection of peptide cleavage subsequent to electrotransfer of the released N-terminal peptide fragment containing antibody epitope [116].

plied to kinase profiling [68, 92, 93] and phosphatase profiling [52] using high density peptide microarrays. A very similar approach was reported using biotinylated zinc(II) chelate phosphate sensor N-(5-(2-(+)-biotin aminoethylcarbamoyl)pyridine-2-ylmethyl)-N,N´,N`-tris(pyridine-2-ylmethyl)-1,3-diaminopropan-2-ol (Phos-tag biotin) [22-24, 27].

Finally, phosphopeptides could be transformed either enzymatically or chemically with high selectivity into labelled (phospho)peptides. Shults *et al.* reported carbodiimide-mediated, selective formation of a covalent bond between a fluorescent dye derivative and the phosphate moiety of phosphopeptides generated by incubation with kinases [94]. Alternatively, Akita *et al.* used ß-elimination to transformed cellulose-surface bound phosphopeptides generated by protein kinase A selectively into dehydroalanine-containing peptides which could be labelled with fluorescence dye derivatives [95]. Enzymatic transformation of the phosphate moiety could be performed using derivatives of ATP substituted at the γ-phosphate residue [96, 97]. Kerman and Kraatz used γ-thio-derivative of ATP to detect transfer of thio-phosphate to surface bound peptides electrochemically using gold nanoparticels [98]. It could be demonstrated that electrochemical detection of kinase mediated transfer of modified phosphate residues is more efficient if electro-active adenosine-5´-[γ-ferrocene] triphosphate is used as co-substrate for protein kinase C [99]. ATP derivatives biotinylated at the γ-phosphate residue were used to detect PKA mediated generation of surface-bound biotinylated phosphopeptides using avidin-stabilized gold nanoparticles amplified by silver deposition [100]. Use of biotinylated ATP derivatives in kinase assays seems to be an interesting alternative to existing technologies [101].

In principle, many other detection principles such as surface plasmon resonance and mass spectrometry, which are comprehensively described in a recent review [7], are possible. Examples have been described for the detection of phosphorylation at peptide microarrays using resonance light scattering [100, 102], surface plasmon resonance [22, 23, 27, 33] and MALDI-TOF mass spectrometry [103-105].

Generally, each assay suitable for kinase profiling on peptide (micro)arrays could be used for analysis of phosphatase-mediated release of phosphate moieties from phosphopeptides, too. Starting surface-bound phosphopeptides could be generated either enzymatically [69] or chemically [69, 106-110]. Detection of phosphatase action on (micro)array bound phosphopeptides by signal decrease subsequent to treatment with anti-phospho-tyrosine antibodies [108-110] or using phospho-specific dyes [52] was demonstrated.

Several different assay principles have been developed to measure protease activity on peptide arrays. Optimal assays lead to increased signal intensity upon substrate cleavage either by reading generated signal in released proteolytic fragment (*see* Fig. **2**) or in still surface bound peptide fragment (*see* Fig. **3**). Several assays of this type have been described: (1) the first assay was developed by Duan and Laursen and is based upon peptide arrays prepared on polyaminoethylmethacrylamide membranes by the SPOT method (*see* Fig. **2A**) [111]. The array comprised all 400 possible dipeptides with an N-terminally coupled fluoresceinyl thiocarbamyl moiety. These peptides were punched out and attached to pins in a microtiter plate lid. Subsequently, they were suspended in wells of a 96-well microtiter plate filled with protease solution. After specified reaction times, the spots were removed in order to quantify the fluorescence dye coupled to the cleaved-off N-terminal peptide fragment. (2) To avoid laborious pin attachment a modified assay involves immersing substrate spots (amino benzoic acid as fluorescence dye) in wells filled with the protease solution (Fig. **2B**) [112,

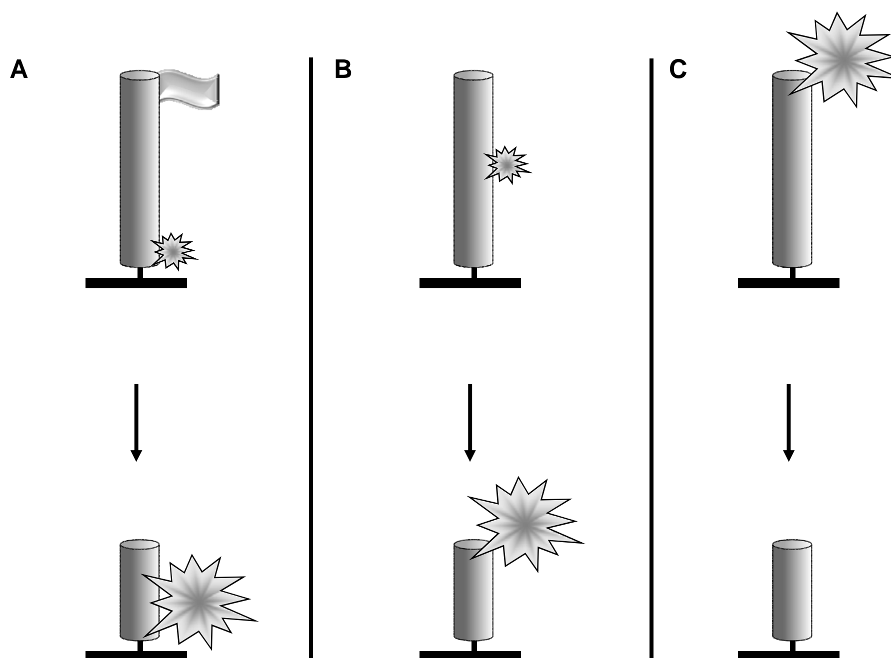

Fig. (3). Detection of Proteolytic Cleavage on Peptide Arrays Reading Surface Bound Fragment. (**A**) Internally fluorescence quenched peptides attached on surface yielding fluorescence increase for array bound peptide fragments subsequent to protease mediated cleavage and removal of peptide fragment containing quenching moiety by washings [46, 114, 115]. (**B**) Immobilized peptides having a fluorogenic group C-terminal to the scissile bond which increases fluorescence subsequent to cleavage by respective proteases [118, 120]. (**C**) Peptides labelled with a fluorescence dye at the terminus distal to the array surface yield decreasing fluorescence signal upon cleavage.

113]. At various times small aliquots are pipetted into new wells and cleavage is quantified using a fluorescence microtiter plate reader. This assay was employed to identify and characterize caspase-3 substrates using substitutional analyses of a known peptide substrate, a peptide scan, combinatorial libraries and randomly generated sets of peptides [114]. The major disadvantage of these two assay principles is that the peptide array has to be dissected, essentially abandoning the benefits of array technologies. (3) This led to the introduction of peptide arrays with internally quenched peptides (*see* Fig. **3A**). Compartmentalization of the cleavage reaction is not necessary and increasing signal intensity is observed. This technique was evaluated using combinatorial peptide libraries and substitutional analyses of substrate peptides incubated with trypsin [115] and subsequently employed to determine the substrate specificity of the integral membrane protease OmpT of *Escherichia coli* [115]. (4) A sophisticated but rather tedious procedure involves peptides coupled to cellulose membranes by their C-terminus and having an antibody epitope tag with a biotinylated lysine residue at the N-terminus (*see* Fig. **2C**). Cleavage releases the N-terminal part of a substrate peptide including the epitope tag and the biotin moiety. This fragment is affinity-blotted onto a streptavidin-coated PVDF membrane and detected via an enzyme–conjugated antibody [116]. A similar procedure was used by Kozlov *et al.* in combination with DNA encoded substrates and DNA-microarrays as sorting device. Potential cleavage site peptides flanked by biotin on one side and penta histidine tag/ DNA tag on the other side were incubated with different proteases or cell lysates. Streptavidin-coated magnetic beads were used to remove non-cleaved peptides and biotinylated cleavage fragments. Remaining members of the library represent His-tagged DNA encoded fragments of cleaved substrates only which could be detected and deconvoluted using anti-penta histidine antibody and fluorescence imaging subsequent to hybridization onto appropriate DNA microarrays [117]. (5) Peptide derivatives containing a substituted fluorogenic group C-terminal to the scissile bond are immobilized on glass slides resulting in peptide microarrays (*see* Fig. **3B**). It was demonstrated that the protease trypsin cleaved the amino acyl-fluorophore bond [85]. A very similar assay principle using longer peptides successfully determined the substrate specificities of trypsin, granzyme B and thrombin employing peptide microarrays on glass slides generated by chemoselective peptide immobilization [118]. (6) Peptides with a fluorescence dye at the free terminus are applied for array-based protease assays with a decreasing signal upon cleavage (*see* Fig. **3C**).

Recently, a novel principle was described for profiling proteolytic activities using semi-wet peptide microarrays and differences in the partition coefficients of peptide substrates and released fluorophores [119]. Lysyl endopeptidase treatment released an environmentally sensitive fluorophore resulting in a blue shift of the emission maximum from 540 nm to 508 nm, along with two-fold higher fluorescence intensity.

In a very elegant experiment, peptidic inhibitors tethered to fluorescence tagged Peptide Nucleic Acids (PNA) were used to profile inhibitor specificity against different cysteine proteases [120, 121]. The peptide nucleic acid tag encodes the structure of the attached peptide derivative and therefore allows spatially addressed deconvolution after hybridization to an oligonucleotide microarray. This approach was extended to the profiling of enzymatic activities of proteases [46, 117, 120] and kinases [47, 94] using PNA/DNA-encoding. Use of oligonucleotides and in particular PNAs to encode libraries was reviewed comprehensively [122].

5. SUBSTRATE IDENTIFICATION

Different scenarios for the identification of kinase substrates are possible. Combinatorial and randomly generated libraries can be applied if no information about potential protein substrates is available. Pioneering work in this field was carried out using low density peptide arrays on cellulose membranes. In this format combinatorial libraries were used to identify substrates for PKA [49, 74, 75, 88], PKG [72, 74, 88], and the budding yeast kinase CDC15 [73]. This approach was also successful using peptide microarrays for p60c-src [31]. A randomly generated library of 1433 tyrosine-containing single peptides on a peptide microarray was used to identify new substrates for c-Abl [18].

While these approaches are suitable for identifying kinase substrate peptides *de novo*, a demanding question in biology is the identification of *in vivo* protein substrates for kinases. Knowledge-based libraries are used to address this problem. In cases where a protein substrate of a certain kinase is known either a selection of peptides containing the potential phospho-acceptor residues of the target protein or a peptide scan of the target protein could be used. For example, three peptides could be identified as substrates for Lyn kinase by using libraries of 15-mer peptides generated from the sequence around each tyrosine residue in PKC [53]. Similary, all tyrosine-containing 13-mer peptides of the cytoplasmic regions of human receptor proteins EphA4 and EphB2 on cellulose membranes were used to define the autophosphorylation sites generated by the EphA4 kinase domain [123, 124] and phosphorylation sites for Abl and c-Src [123]. Decapeptide sequences derived from the cytoplasmic domains of C-CAM revealed a single specific phosphorylation site for PKC [54]. Overlapping peptide scans were used to determine PKA phosphorylation sites in myelin basic protein [19], GSK-3ß phosphorylation sites in p65 NF-kappaB [125], and the autophosphorylation sites as well as sites for CK2 mediated phosphorylation in the tyrosine kinase Tie2 [19].

Even though initial proof-of-concept experiments with 18 cellulose membrane bound peptides derived from protein sequences phosphorylated by PKC *in vivo* [58] were successful [126], the full power of the knowledge-based approach emerged when applied to high density, high content peptide microarrays. Peptide microarrays displaying the sequences of 710 human annotated phosphorylation sites revealed peptide substrates for NEK6 [16], Abl [17], and PKA/CK2/GSK3 [19]. More advanced libraries were used for CK2 (11096 peptides from cytoplasmic domains of human membrane proteins and 2304 human annotated phosphorylation sites) [17, 62], PDK1 (1394 peptides derived from the activation loops of human kinases) [19] and CK2 or Gsk3 (17181 peptides representing overlapping peptides covering complete proteome of cytomegalovirus) [55]. Peptide substrates identified in the microarray experiments were superior to known peptide substrates for PDK1 and NEK6, as demonstrated by determination of catalytic constants in solution phase experiments [16, 19].

Microarrays displaying several hundred peptides derived from human phosphorylation sites were used to fingerprint differences in the substrate specificities of oncogenic Bcr-Abl and NUP214-Abl fusion proteins, both yielding aberrant tyrosine kinase activation, and to analyse the sensitivity of these enzymes against the inhibitor imatinib (Glivec) [127]. Moreover, microarrays displaying more than 1000 peptides derived from human phosphorylation sites enabled the identification of novel substrates for receptor tyrosine kinase Flt3 [128], mitogen activated protein kinase kinase 8 [129], and transmembran serine/threonine kinase KPI [130].

An additional application of peptide microarrays is the detection of priming phosphorylation events. In such processes substrates for a certain kinases are generated upon previous phosphorylation with another kinase on different phospho-acceptor amino acids of the substrate. This was shown for the system CK2 as priming kinase and GSK3 as second kinase with a library of 694 annotated human phosphorylation-site peptides where all corresponding CK2 monophosphorylated derivatives were produced by incorporating phospho-amino acid building blocks during synthesis [19]. Similar approach was described by Coba et a. using kinases GSK3, CaM-KII, Erk2, ROCK-II, Fes and JNK3 [131].

There is some evidence for a minimal eukaryotic phosphoproteome coming from experiments with microarrays displaying peptides derived from human phosphorylation sites and cell lysates from different organisms like *P.pastoris, T.aestivum, C.albicans, A.thaliana, F. solani, M.musculus* and *H.sapiens* [64]. It could be demonstrated that phosphorylation profiles show a large overlap despite the divergence of the protein kinases on the primary structure level. These findings are underlined by the facts that peptide microarrays with human sequences were successfully used to characterize the substrate specificity of mitogen activated protein kinases from tomato [59], Big mitogen-activated protein kinase 1 from mice [61], protein kinase 7 from *P. falciparum* [132], proline-directed kinase PknG from *S.aureus* [133] and tyrosine kinase activities in zebrafish embryos [25, 26].

6. SUBSTRATE OPTIMIZATION

Using cAMP- and cGMP-dependent protein kinases (PKA and PKG) as model enzymes, Tegge *et al.* [74] applied peptide arrays on cellulose membranes to identify substrates from combinatorial libraries with the format Ac-XXXO_1O_2XXX. X represents mixtures of all 20 proteinogenic amino acids, while O_1 and O_2 represent individual amino acids defined for one spot but varying between different spots in a library. Applying all 20 naturally encoded amino acids at these defined positions will result in 20 x 20 = 400 spots or peptide mixtures. After a first screening round of this initial library the best two amino acids at positions 1 and 2 are retained throughout the optimization cycles and two new positions are defined. This strategy led to the identification of a new, very efficient peptide substrate for PKG [134]. Extending this approach to longer peptides yielded substrates highly specific for PKG [71]. Analysis of these results revealed a central role for PKG in the modulation of vascular contractility [72, 75]. Different combinatorial libraries led to the deconvolution of substrate sequences for PKA and type I and II TGF-α receptor kinases [49].

Toomik and Ek [58] used the SPOT technology to synthesize an optimization library for PKC substrates, with the flanking residues of a known substrate substituted by different amino acids. Similar experiments led to optimized substrate sequences for the calcium-dependent kinase from maize seedlings [77].

A different approach is a complete substitutional analysis [135]. Substitutional analysis of a histone H3 derived peptide on cellulose membranes led to the discovery of DYRKtide, an efficient substrate for DYRK1a [76]. On peptide microarrays substitutional analyses were used to determine the substrate requirements of NEK6 [16] and p60c-src [31]. Moreover, iterative substitutional analyses resulted in optimized substrates for murine Plk4 [136] and EphA4 receptor tyrosine kinase [123].

7. DETERMINATION OF REACTIVITY PROFILES

The possibility to measure multiple enzymatic reactions in parallel with minute amounts of sample on peptide microarrays opens the way to analyse the sum of activities of enzyme classes in biological fluids like serum, cell lysates or lysates from complete organisms, like zebrafish embryos [25, 26]. Several applications of kinase reactivity profiling on peptide microarrays are described. Response prediction of multitarget kinase inhibitors became feasible using peptide microarrays in combination with lysates from cancer cell lines and xenograft tumors [137]. Additionally, synergistic effects on multiple carcinoma cell lines could be demonstrated for the kinase inhibitors lapatinib and pazopanib using a similar approach [138]. Efficacy of inhibitors could be tested with peptide microarrays. Schrage *et al.* were able to demonstrate by treatment of peptide microarrays with lysates from untreated or imatinib-tretated GIST882 cells, a gastrointestinal stromal tumor cell line carrying a mutation in KIT, that this inhibitor targets the Ras/Raf/MEK/ERK pathway *in vivo*. Additionally, it could be shown that resulting kinase reactivity patterns are different for chondrosarcoma cell cultures and colorectal carcinoma cell lines [139]. Roorda *et al.* introduced kinase reactivity profiling on peptide microarrays as powerful tool in functional studies by analysing effects of small molecule tyrosine kinase inhibitor PTK787/ZK222584 on kinases involved in cell cycle control [140].

Additionally, this approach was used to analyse the effects of treatment with glucocorticoid [66, 67], cyclooxygenase-2 inhibitor Celecoxib [141], spongistatin [142], lipopolysaccharide [63], ACE inhibitor Ramipril [143] and anti-CD45RB antibody [144] on kinase reactivity profiles of the respective cell lysates. Bowick *et al.* used kinase reactivity profiling to characterize the host cell response to lethal arenavirus infections [145]. In zebrafish embryos effects of morpholino-mediated protein knock down as well as changes in the embryonic development on phospho-tyrosine signalling were analyzed using tyrosine kinase reactivity profiling in combination with peptide microarrays displaying several hundred tyrosine phosphorylation-site derived peptides [25, 26]. Finally, enzyme reactivity profiles could be indicative for the status of a cell. Sikkema *et al.* identified characteristic tyrosine kinase reactivity profiles in 29 pediatric brain tumors [146] and van Baal *et al.* were able to detect three unique kinase reactivity profiles in biopsies of 27 Barett´s esophagus patients as compared to normal squamous esophagus [65]. It could be expected that extension of this methodology to more features on the microarrays and to other enzymatic activities, like lysine side chain acetylation and methylation, will generate novel biomarkers for cancer and autoimmune diseases.

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