Peptide Arrays for Kinase Profiling

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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 the molecular origins of disease and for potentially developing tools for therapeutic intervention. The discovery of more than 500 such enzymes in the human genome has 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 arrays, which have proved to be powerful tools for the rapid delineation of molecular-recognition events.

Here, we describe the production principles, library types and assay strategies used for kinase profiling on peptide arrays. The second part summarizes various peptide array applications in kinase research.

2. Array Production and Assay Principles

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 presynthesized peptide derivatives. 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 that permits extreme miniaturization of array formats (several hundred peptides per cm^2); however, it involves sophisticated and rather tedious synthesis cycles. A major problem is the novel set of chemistries that reduce the quality of the surface-bound peptides. They results in false positive (if an impurity is active) and/or false negative results (if the target peptide sequence was not synthesized at all). An interesting alternative is the use of photogenerated acids in combination with standard Boc chemistry.[2–4] Nevertheless, the use of photolithografic masks combined with solid-phase peptide synthesis is relatively labor intensive.

Alternatively, the SPOT synthesis concept developed by Ronald Frank consists of the stepwise synthesis of peptides on planar supports, such as functionalized cellulose membranes or aminated polypropylene, applying standard Fmoc-based peptide chemistry.[5–7] SPOT synthesis is technically very simple and flexible and does not require any expensive laboratory automation or synthesis hardware. The resulting spot size is defined by the dispensed volume as well as the physical properties of the surface used. The degree of miniaturization when using SPOT synthesis (9–16 spots per cm^2) is significantly lower than for peptide microarrays. SPOT synthesis has been reviewed extensively.[8–18]

In fact, due to the simplicity of the SPOT concept, all published applications of in situ synthesized peptide arrays for kinase profiling are based on SPOT synthesis on flexible membranes (see references in Table 1).

When large numbers of peptide arrays with the same sequences are required, immobilization of presynthesized 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 peptide.

One intrinsic advantage of using chemoselective reactions is the introduced reactivity-purification step. The resulting target peptide derivative is contaminated by acetylated truncated sequences only if the chemical moiety mediating the chemoselective reaction with the appropriately modified surface is attached to the N terminus of the growing peptide and the peptide synthesis protocol is modified by introduction of capping steps. Deposition of this mixture results in a covalent bond forming exclusively between the target peptide derivative and the surface. The chemically "inert" truncated sequences can be simply removed during subsequent washing steps. Thus, chemoselective reactions allow the generation of peptide arrays displaying purified peptides that are free of truncated sequences.

Different chemoselective reactions were used for peptide microarray preparation in connection with kinase experiments. An aldehyde function at the surface of glass slides in combination with aminooxyacetyl moieties in the peptides[14,19–25] or cysteinyl residues[21,26] was used for the preparation of peptide microarrays on glass slides. It could be demonstrated that

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Table 1. *Bibliography of applications that make use of peptide arrays for kinase profiling and substrate identification. The entries are arranged according to the publication year and then alphabetically by author surnames (see references).*

Screening Molecule Kinases and Phosphatases	Library	Technology[a]	Ref.
Protein kinase A (PKA); cytoplasmic (kinase) domains of transforming growth factor β (TGFβ) type I and II receptors; γ^{32}P-ATP	several combinatorial libraries with fixed amino acids and randomized positions (17 amino acids excluding Cys, Ser, Thr) at different positions	SODA	[33]
Protein tyrosine kinase Lyn, antiphosphotyrosine antibody and POD-labeled second antibody for detection	panel of 23 PKCδ-derived 15-mer peptides	SPOT	[36]
Catalytic subunit of protein kinase A- or cGMP-activated protein kinase G and γ^{32}P-ATP	combinatorial library of the type $XXXO_1O_2XXX$ and iterative deconvolution libraries	SPOT	[47]
–anti-porcine tubulin–tyrosine ligase (TTL) mab 1D3	–substitutional and truncation analysis of a TTL-derived peptide; combinatorial library (XXB_1B_2XX) and deconvolution libraries	SPOT	[53]
–streptavidin–alkaline phosphatase	–combinatorial library (XXB_1B_2XX) and deconvolution libraries		
–cAMP-dependent protein kinase	–combinatorial library (XXB_1B_2XX) and deconvolution libraries		
–casein kinase I (CKI) or II (CKII) or protein kinase C (PKC) or catalytic subunit of protein kinase A (PKA) and γ^{32}P-ATP	–peptides derived from the phosphorylation sites RRASVA, QKRPSQRAKYL, DDDDEESITRR, DDDSDDDAAAA	SPOT	[61]
–potato acid phosphatase	–RRASS*VA, QKRPS*QRAKYL, DDDDEES*ITRR, DDDS*DDDAAAA (S* = phosphoserine)		
Protein kinase A (PKA), protein kinase C (PKC), casein kinase I (CKI), casein kinase II (CKII) and γ^{32}P-ATP	panel of human phosphorylation sites	SPOT	[40]
–^{125}I-calmodulin	–phosphorylated and unphosphorylated peptides derived from cell–cell adhesion molecule (C-CAM) isoforms S and L and single-site substitution analogues	SPOT	[37]
–porcine protein kinase C β and γ^{32}P-ATP	–partial peptide scans of the C-CAM isoforms S and L		
Androgen receptor protein kinase and γ^{32}P-ATP	panel of kinase substrates known from literature	SPOT	[54]
Enzyme I of bacterial sugar phosphotransferase system and ^{32}P-phospho*enol*pyruvate	combinatorial library of the type $XXXXXO_1HO_2XXXXX$ and iterative deconvolution libraries	SPOT	[42]
Catalytic subunit of protein kinase A or cGMP-activated protein kinase G and γ^{32}P-ATP	combinatorial libraries of the type $XXXO_1O_2XXX$ and $XXXRRO_1O_2X$	SPOT	[50]
	combinatorial libraries of the type $O_1KARKKSNO_2$, O_1O_2T-QAKRKKSLA, $O_1O_2KATQAKRKKSLA$, $TQAKRKKSLAO_1O_2$, and $TQAKRKKSLAMAO_1O_2$	SPOT	[44]
	combinatorial library of the type $XXXO_1O_2XXX$ and deconvolution libraries: $XXXRKO_1O_2X$, $XRKKKO_1O_2X$, $O_1RKKKKKO_2$, $LRKKKKKHO_1O_2$, and $O_1O_2LRKKKKKH$	SPOT	[43]
GST-Dyrk1A-Δ(500–763) and γ^{32}P-ATP	partial substitutional analysis of RRRFRPASPLRGPPK	SPOT	[48]
Maize Ca^{2+}-dependent protein kinase (CDPK-1) and γ^{32}P-ATP	partial substitutional analysis of LARLHSVRER	SPOT	[49]
Protein kinase A (PKA), casein kinase II (CKII), p42-MAP kinase (Erk2) and γ^{32}P-ATP	kinase substrates kemptide and Elk1; protein kinase inhibitor 2	DIPP	[34]
–$p60^{c\text{-}src}$ protein tyrosine kinase and γ^{32}P-ATP	–Ttds-EEIYGEFF	DIPP	[21]
–strepavidin-Cy3 conjugate; avidin-Cy5 conjugate	–biotin, HPYPP and WSHPQFEK		
–anti-human insulin mab HB125 and anti-mouse IgG-Cy5 conjugate	–biotin, wGeyidvk, pqrGstG, WSHPQFEK and YGGFL		
–WEHI-231 cells and negative control cells	–wGeyidvk		
Practical protocols to measure phosphorylation of peptides on peptide arrays prepared by SPOT synthesis exemplified by the kinase CKII		SPOT	[51]
Cyclic GMG-dependent protein kinase (phosphorylation γ^{32}P-ATP)	$XXXRKB_1B_2X$ (deconvolution libraries described but data not shown)	SPOT	[45]
^{32}P-labeled cyclic GMG-dependent protein kinase (binding)	$B_1RKKKKKB_2$ (preceding libraries described but data not shown)		
–GST-protein tyrosine phosphatase 1B; mixture of antiphosphotyrosine antibodies, POD-conjugated anti-mouse antibody	–15-mer phosphotyrosine-containing peptides derived from human insulin receptor; degenerate SPOT library of general structure $AABX_1ZX_2BAA$ (Z = phosphotyrosine, B = mixture of 20 natural amino acids, X = defined amino acids) and peptides derived from human STAT 5A containing both phosphotyrosine and phosphothreonine residues	SPOT	[62]
–GST-protein tyrosine phosphatase β; mixture of antiphosphotyrosine antibodies, POD-conjugated anti-mouse antibody	–15-mer phosphotyrosine-containing peptides derived from human Tie2 receptor containing additional phosphoserine or phosphothreonine residues		
–GST-fusion protein of substrate-trapping mutant of protein tyrosine phosphatase 1B (D181A), radioactively labeled by incubation with protein kinase A in the presence of γ^{32}P-ATP	–15-mer phosphotyrosine-containing peptides derived from human insulin receptor; degenerate SPOT library of general structure $AABX_1ZX_2BAAA$ (Z = phosphotyrosine, B = mixture of 20 natural amino acids, X = defined amino acids), progressive alanine substitution of MTRDIYETDYZRKGG (Z = phosphotyrosine), alanine scan of TRDIYETDYZRKGGKGL, substitutional analysis for X in MTRDIYETDXZRKGG (Z = phosphotyrosine)		
c-Src and γ^{32}P-ATP or antiphosphotyrosine antibody	panel of known kinase substrates	DIPP	[31]
c-Src and γ^{32}P-ATP	c-Src substrate	DIPP	[63]

Table 1. (Continued)

Screening Molecule Kinases and Phosphatases	Library	Technology[a]	Ref.
p60 and FITC-labeled antiphosphotyrosine antibody	YIYGSFK, ALRRASLG, KGTGYIKTG, and monophosphorylated derivatives	DIPP	[26]
PKA or p60 and FITC-labeled antiphosphotyrosine or antiphosphoserine antibody	YIYGSFK, ALRRASLG, YIYGSFK, and monophosphorylated derivatives	DIPP	[28]
NEK6 kinase and γ^{32}P-ATP	human annotated phosphorylation sites and mutational analysis of GLAKSFGSPNRAY	DIPP	[22]
Protein tyrosine kinase ABL and γ^{32}P-ATP or FITC-labeled antiphosphotyrosine antibody	collection of 720 peptides (13-mer) derived from human annotated phosphorylation sites (databases SwissProt and Phosphobase)	DIPP	[14]
–GST-ERK-2 or GST-MEK-EE, antiphospho-Elk-1 antibody, POD-labeled anti-rabbit antibody	–double peptide synthesis of ELK1 substrate peptide FWSTLSPIAPR, D-loop peptide KGRKPRDLELP and control peptide MNGGAANGRIL	SPOT	[41]
–GST-fusion protein–protein tyrosine phosphatase 1B;antiphosphotyrosine antibody, POD-conjugated anti-mouse antibody	–double peptide synthesized SPOTs containing the PTP-1B substrate IYETDYZRKGG (Z = phosphotyrosine) and on the second site a scan of 11-mer overlapping peptides derived from the cytoplasmic domain of insulin receptor		
–GST-fusion protein of substrate-trapping mutant of protein tyrosine phosphatase 1B (D181A), radioactively labeled by incubation with protein kinase A in the presence of [γ^{32}P]ATP	–double peptide synthesis of insulin receptor-derived substrate peptide IYETDYZRKGG (Z = phosphotyrosine) and binding motif for YAP WW1 domain and p53 binding protein-2 (YPPYPPPPYPS)		
PKA and Abl; Pro-Q Diamond phospho-specific stain or FITC-labeled antiphosphotyrosine antibody for detection	kemptide, p60 c-src (521–533), delta sleep-inducing peptide (DSIP), phosphoDSIP, CamKII peptide (GS1–10), different proteins	DIPP	[52]
GST-proteins of substrate-trapping mutants of tyrosine phosphatases (PTP-H1, SAP-1, TC-PTP, PTP-1B) radioactively labeled by incubation with protein kinase A in the presence of γ^{32}P-ATP	7 peptides (14-mers) derived from human GHR together with the appropriate phosphotyrosine-containing derivatives	SPOT	[64]
Tyrosine kinase p60 c-scr and FITC-labeled antiphosphotyrosine antibody	deletion, alanine-scanning, positional scanning, and full combinatorial mixture libraries of CGG-YIYGSFK (p60 c-src substrate)	DIPP	[29, 30]
–protein kinase A and tyrosine kinase Abl and Cy5-labeled antibodies	–kemptide–leptin fusion protein and Abl-peptide–leptin fusion protein	DIPP	[35]
–phospho-specific antibodies MPM2, 3F3/2, PY20	–complete L-amino acid substitutional analysis of AXXXX[pS/pT]-XXXXA and AXXXX[pYT]XXXXA	SPOT	[46]
–GST fusions of GRB-SH2, SHP2-CSH2, GRB7-SH2, GRB 10-SH2	–complete L-amino acid substitutional analysis of AXXXX-[pYT]XXXXA		
–protein kinase A (PKA), Cdc 15	–complete L-amino acid substitutional analysis of $AX_1X_1X_1X_1[S/T]$-$X_1X_1X_1X_1A$ X = L-amino acids except for Cys, X_1 = L-amino acids except for Cys, Ser, Thr		
Tyrosine kinase Abl and CKII together with FITC-labeled antiphosphotyrosine antibody and γ^{32}P-ATP, respectively	collection of more than 13 000 peptides (13-mer) derived from human proteins	DIPP	[23]
FITC-labeled antiphosphotyrosine antibody	2923 phosphopeptides (13-mer) derived from human proteins		
Tyrosine kinase Abl and γ^{32}P-ATP	1433 randomly generated 15-meric peptides	DIPP	[24]
Kinases PDK1, Tie2, CKII, PKA GSK3, and [γ^{32}P]ATP	peptide scans trough MBP and Tie2, 720 human annotated phosphorylation sites (13-mer peptide, 2923 phosphopeptides (13-mer) derived from human proteins, 1394 peptides derived from activation loops of human kinases	DIPP	[25]
Kinases Src, PKA, PKG, CaMKII, CKI, Abl, Erk	7 peptides, each of a known specific substrate for one of the kinases	DIPP	[7]
Kinases and phosphatases contained in lysates of IL1-stimulated HeLa cells	peptide scan through p65 NF-κB, selected peptides from the scan and controls with systematic mutation of Ser, Thr, Tyr to nonphospho-acceptor residues	SPOT	[19]
Kinases and phosphatases in lysates of lipopolysaccharide-stimulated and -unstimulated human peripheral blood mononuclear cells	192 nonapeptides providing consensus sequences across the mammalian kinome	DIPP	[20]

[a] SPOT = spot synthesis of peptides; DIPP = directed immobilization of presynthesized peptides; SODA = synthesis on defined areas.

native chemical ligation, introduced by Dawson et al.[27] is well suited for effective attachment of kinase substrate peptides containing an N-terminal cysteine residue to thioester-modified glass slides.[28–30] A more sophisticated reaction for oriented immobilization of peptide derivatives was introduced by Houseman et al.[31] 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.[31]

The surface-bound peptides' accessibility to the proteins or enzymes used in screening has also been identified as a critical factor. Insertion of a spacer between the peptide and the surface is an effective way to circumvent this potential problem.

Such spacers can 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.[32] For protein tyrosine kinase $p60^{c\text{-}src}$, it was demonstrated that incorporation of 1-amino-4,7,10-trioxa-13-tridecanamine succinimic acid as a spacer moiety was necessary for effective phosphorylation of glass-surface-bound peptides.[21] Moreover, insertion of hydrophilic dextran structures between the surface and the presented peptides proved necessary for efficient kinase substrate interaction.[33]

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 the amino acid side chains of the albumin protein.[34] Alternatively, substrate peptides fused genetically to the C terminus of human leptin were immobilized onto aldehyde-modified glass slides.[35] By using this method, sensitivity was increased by three orders of magnitude compared to other microarray approaches with PKA and leptin–kemptide fusions.

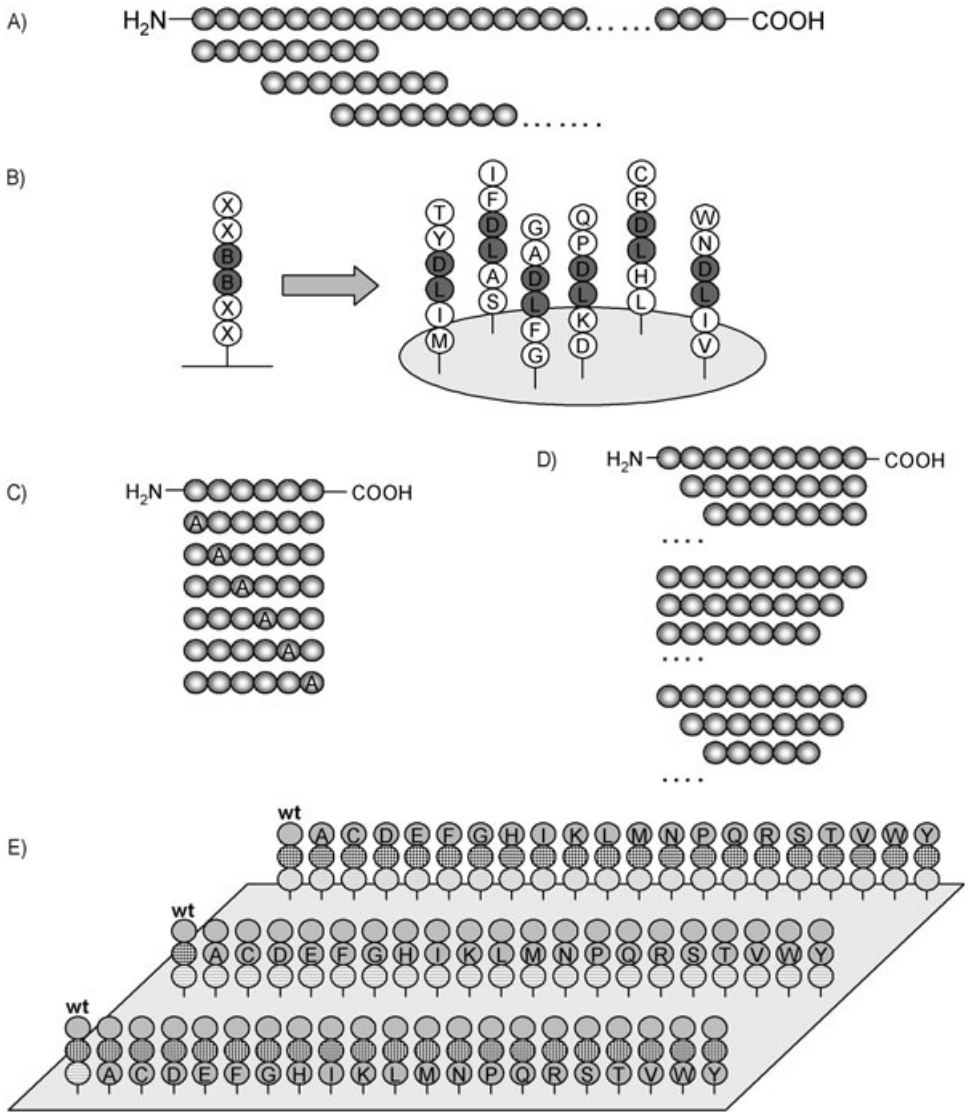

Figure 1. *A) The amino acid sequence of the protein under investigation is used to generate short linear overlapping octamer peptides shifted by three amino acids (peptide scan). B) Peptide mixtures with defined positions B and randomized positions X. C) Amino acid substitution scan (alanine scan) of a hexamer peptide. D) Truncation library with N-terminal, C-terminal, and bidirectional stepwise truncations. E) Complete substitutional analysis of a trimer peptide.*

Library types

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

The first type of knowledge-based library is of particular interest when a kinase's protein target is known. Identification of the actual phospho-acceptor residue is achieved by scans of overlapping peptides ("peptide scans") derived from the protein's sequence (Figure 1 A). Alternatively, libraries of peptides that only cover the sequence around each potential phospho-acceptor residue have been used.[36,37] The availability of high-density peptide microarrays has enabled the systematic extension of this approach in a "proteomics-like manner", such that addressing groups of proteins comprising the cytoplasmic domains of all human membrane proteins has resulted in more than 11 000 peptides,[23] or covered the activation loops of all human kinases.[25] In a similar approach, the sequences of experimentally identified phosphorylation sites taken from databases (i.e. Swissprot[38] and Phosphobase[39]) and the literature were comprehensively evaluated on peptide arrays.[14,20,25,40] The use of such peptides increases the probability of finding substrates for a given kinase, since each peptide's sequence is known to be phosphorylated by a kinase at least in the context of the natively folded protein. One problem associated

with knowledge-based libraries is the uncertainty as to which residue is phosphorylated when multiple phospho-acceptor sites occur in one individual peptide. When using peptide scans, this information can be extracted from increasing and decreasing signal intensities appearing along consecutive peptides. This is illustrated by scanning myelin basic protein phosphorylated by cAMP-dependent kinase, as shown in Figure 2.

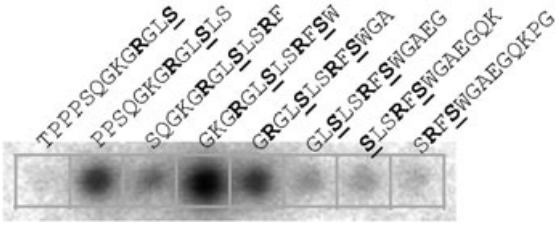

Figure 2. *Section of the Phosphor Image of a peptide microarray displaying a scan of overlapping peptides derived from the myelin basic protein MBP (13-mers overlapping by 11 amino acids) after phosphorylation with PKA in the presence of [^{32}P]ATP.[25] Serine residues shown to be phosphorylated in the native MBP[60] are written in bold and underlined. The key residue arginine is also shown in bold. The strongest signal is observed when both phosphorylation sites are in the central region of the substrate. When the phosphorylation site is positioned at one of the peptide termini, phosphorylation is not effective.*

Statistical analysis and alignment of the sequences proved to be useful in the case of collections of annotated phosphorylation sites.[23] Identification and alignment of key residues in the different substrate peptides allows a reliable assignment of the actual phospho-acceptor residue(s).

An extension of the knowledge-based-libraries concept is the introduction of post-translational modifications (e.g., phosphorylation, methylation, or acetylation) within the substrate sequences. This more adequately mimics the natural environment in which phosphorylation occurs and allows the detection of peptides that become substrates only after an initial or priming modification event. Such modifications can be introduced enzymatically after chemical synthesis of the unmodified peptides or chemically by using modified building blocks during the course of synthesis.

Another type of knowledge-based library allows the mapping of protein interactions involving two discontinuous components that are far apart in the primary polypeptide structure but form a composite phosphorylation site in the natively folded protein. Two separate peptides are synthesized independently by a double-peptide-synthesis method on a single spot so as to allow the detection of synergistic pairs of peptides.[41]

For de novo detection of kinase substrates both combinatorial approaches and randomly generated libraries of single peptides have proved to be useful. Combinatorial libraries have one or more defined amino acid position(s) and a number of randomized or degenerated positions.[29,33,42–46] Only one particular amino acid is introduced at the defined positions, while a mixture of amino acids is introduced at the randomized positions; this results in a sublibrary of different sequences in each single spot (Figure 1B). 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. A very high diversity can be achieved due to the huge number of different peptides. 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 deconvoluted by 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 guaranteed when using peptide microarrays with a low concentration of peptide per spot on planar surfaces.[29]

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. 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, the random libraries can be biased by introducing defined positions derived from the consensus sequences. Randomly generated libraries show a higher sequence diversity compared to knowledge-based libraries that are biased toward known kinase substrates. This can be an advantage in searching for selective substrates for closely related kinases.

Substrate characteristics, that is, key interaction residues, can be deduced from all these library types by using statistical analysis, provided that the number of identified substrates is high enough.[23,24,43–47] Alternatively, different library types based on single substrate sequences, such as alanine scans (Figure 1C), deletion (Figure 1D),[29] and substitutional libraries (Figure 1E),[22,40,48,49] permit comprehensive substrate characterization.

Assays and detection

Two different detection principles are applied to detect phosphorylated peptides on peptide arrays. One way is to incorporate a radioactive label during the phosphorylation reaction by using [γ-$^{32/33}$P]ATP (Figure 3A). Subsequently, quantification of incorporated radioactivity is achieved by using either a Phosphor Imager or X-ray films, or alternatively photographic emulsions that deposit silver grains directly onto the glass surface.[34] 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[19,50,51] and peptide microarrays.[20] However, for reasons of operational safety, ease of handling, and waste disposal, radioactive detection methods are increasingly avoided. Additionally, the extremely high peptide loading on cellulose membranes (up to 10 nmol mm^{-2}) may lead to unspecific binding of negatively charged ATP to positively charged peptides. False positives can only be ruled out by including control peptides lacking the phospho-acceptor moiety.[19] The approximately

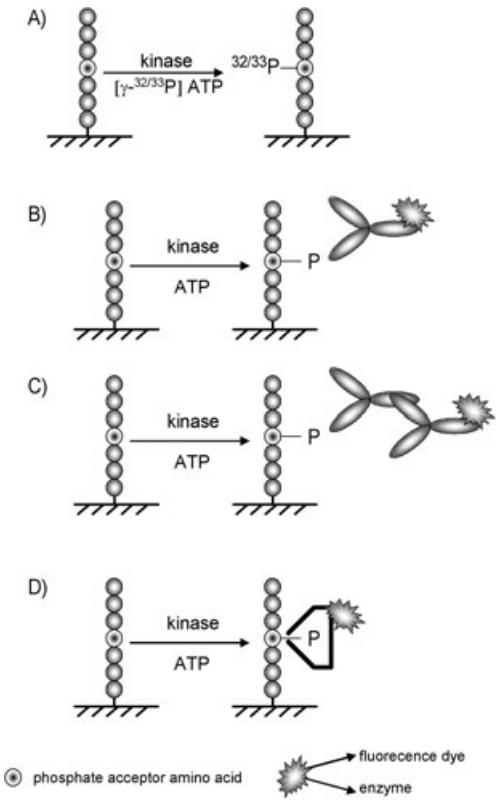

Figure 3. *Assay principles for the detection of peptide phosphorylation on peptide arrays. A) The array is incubated with the kinase of interest in the presence of [γ-^{32} or ^{33}P]ATP, and autoradiography is used for detection. Alternatively, phosphorylation is measured with B) a labeled antiphosphoamino acid antibody or with C) an antiphosphoamino acid antibody in combination with a labeled secondary antibody. D) Phosphoamino acid detection with a labeled phospho-amino acid chelator.*

1 million-times-lower peptide loading on glass slides avoids unspecific binding of ATP, thus making peptide microarrays on glass surfaces perfectly suited for radioactive readout.

Alternatively, phosphopeptides can be detected by using phospho-specific antibodies (Figure 3 B, C) or chelators (Figure 3 D) that are labeled with a detection moiety. The detection moiety can be a fluorescent label, such as fluoresceine,[26,28] or an enzyme, for example horseradish peroxidase, generating a chemiluminescent signal in combination with an appropriate substrate.[41] The detection moiety can be coupled either directly to the antiphosphoamino acid antibody (Figure 3 B)[14,29] or to a secondary antibody (Figure 3 C).[31,41] A recent study assessed the quality of antiphosphoamino acid antibodies as detection tools compared to the radioactive detection method generally recognized as the gold 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.[23] Only monoclonal antiphosphotyrosine antibodies showed reliable results with no detectable binding to non-phosphorylated amino acids, only a limited false-negative rate, and few cross-reactive binding events to phosphoserine or phosphothreonine residues. Antiphosphoserine antibodies, however, had an extremely high false-negative rate, while antiphosphothreonine antibodies showed significant cross reactivities for peptides with phosphotyrosine. In conclusion, the detection of phosphopeptides by using antibodies seems to be limited to the detection of phosphotyrosine residues, at least until suitable antiphosphoserine and antiphosphothreonine antibodies are available.

Alternatively, one can use phosphoamino acid chelators coupled to a detection moiety (Figure 3 D). Martin et al. described the Pro-Q Diamond phosphosensor dye (Molecular Probes, Inc., Eugene, US), which recognizes phosphopeptides with remarkably little cross reactivity and a low false-negative rate.[52] Moreover, proof-of-concept examples have been described for detecting kinase-mediated peptide phosphorylation on microarrays by using surface plasmon resonance[31] or MALDI-TOF mass spectrometry.[7]

3. Applications

Peptide microarrays are predominantly used for the identification and optimization of kinase substrates. The identified substrates are a prerequisite for high-throughput screening in drug discovery. Additionally, the information about substrates and substrate specificity can deliver valuable hints for integrating orphan kinases into the signal-transduction network.

Multiplexed treatment of peptide microarrays with cell lysates generates snapshots of the actual phosphorylation equilibrium within cells and reflects the activity of kinases and phosphatases.[19,20] This should facilitate novel diagnostic concepts based on phosphorylation fingerprints.

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 with low-density peptide arrays on cellulose membranes. In this format, combinatorial libraries were used to identify substrates for PKA (Figure 1 B),[33,43,46,47,50] PKG,[45,47,50] and the budding yeast kinase CDC15.[46] This approach was also successful when using peptide microarrays for p60c-src.[29] A randomly generated library of 1433 tyrosine-containing single peptides on a peptide microarray was used to identify new substrates for c-Abl.[24]

While these approaches are suitable for identifying kinase substrate peptides de novo, a demanding question in biology is the identification of kinases' natural protein substrates in order to integrate novel kinases into their biological context and signal-transduction networks. The data generated from combinatorial or random libraries are of limited use for answering such questions. The resulting substrate sequences are usually not found in nature, and natural substrates can only be deduced by looking for similar naturally occurring sequences. Knowledge-based libraries are therefore used to overcome this problem.

In cases in which a protein substrate of a certain kinase is known and the aim is to identify the actual site of phosphorylation within the target protein, two straightforward approaches are to use a selection of peptides containing the potential phospho-acceptor residues of the target protein or a peptide scan of the target protein. 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 PKCδ.[36] Decapeptide sequences derived from the cytoplasmic domains of C-CAM revealed a single specific phosphorylation site for PKC-β.[37] Overlapping peptide scans were used to determine PKA phosphorylation sites in myelin basic protein (Figure 2) and the autophosphorylation sites as well as sites for CK2 mediated phosphorylation in the tyrosine kinase Tie2.[25]

Even though initial proof-of-concept experiments with 18 cellulose membrane-bound peptides derived from protein sequences phosphorylated by PKC in vivo[40] plus later studies were successful[53,54] the full power of the knowledge-based approach emerged when applied to high-density, high-content peptide microarrays.

Peptide microarrays displaying sequences of human annotated phosphorylation sites revealed peptide substrates for NEK6,[22] Abl,[23] PKA/CK2/GSK3,[25] and more than 85 other kinases (Figure 4). More advanced libraries were used for CK2 (11 096 peptides from cytoplasmic domains of human membrane proteins and 2304 human annotated phosphorylation sites)[23] and PDK1 (1394 peptides derived from the activation loops of human kinases).[25] 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.

An additional application of these microarrays is the detection of priming phosphorylation events. In such processes, substrates for 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 GSK3β as second kinase with a library of 694 annotated human phosphorylation-site peptides in which all corresponding CK2 monophosphorylated derivatives were produced by incorporating phosphoamino acid building blocks during synthesis.[25]

Substrate optimization

From the beginning, the use of peptide arrays in kinase research focused on kinase substrate optimization in terms of substrate efficiency and selectivity. Using cAMP- and cGMP-dependent protein kinases (PKA and PKG) as model enzymes, Tegge et al.[47] applied peptide arrays on cellulose membranes to identify substrates from combinatorial libraries with the format Ac-XXX12XXX. (X represents mixtures of all 20 proteino-

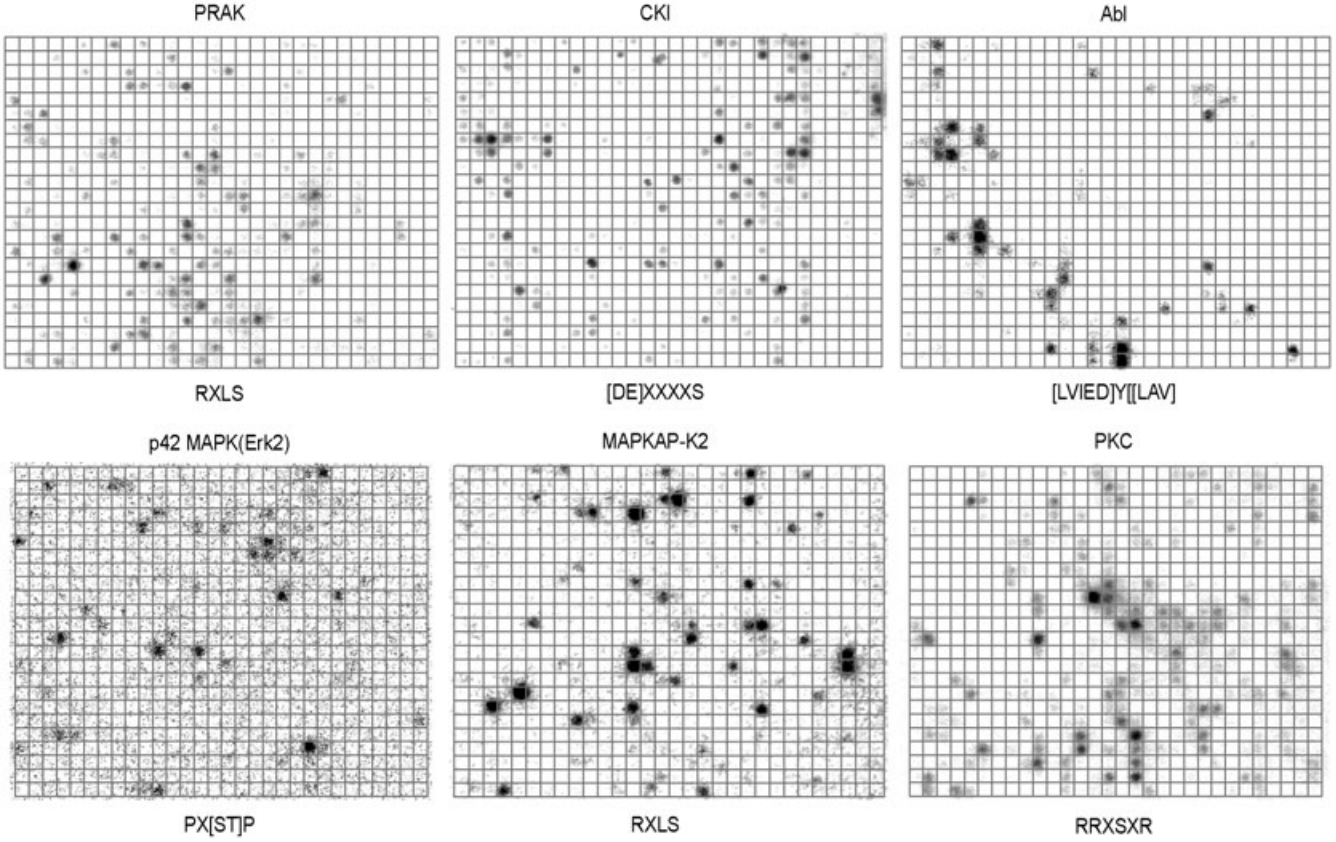

Figure 4. *Profiling of six different kinases by using peptide microarrays displaying 710 annotated human phosphorylation site-derived peptides. Phosphor images obtained after incubation with the kinase in the presence of [γ-^{32}P]ATP are shown together with the consensus sequence determined by statistical analysis of the results.*

genic amino acids, while 1 and 2 represent individual amino acids defined for one spot but varying between different spots in a library.) Incorporation of all 20 naturally encoded amino acids at these defined positions results in $20 \times 20 = 400$ 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 procedure is repeated iteratively until each position is refined and one single peptide sequence per spot is obtained. This strategy led to the identification of a new, very efficient peptide substrate for PKG, and selected a PKA substrate with properties very similar to the known kemptide peptide.[55] Extending this approach to 12- and 14-meric peptides yielded highly specific substrates for PKG.[44] Substituting the phospho-acceptor residue by alanine resulted in specific inhibitory peptides. Analysis of these results revealed a central role for PKG in the modulation of vascular contractility.[43,45]

Different combinatorial libraries led to the deconvolution of substrate sequences for PKA and type I and II TGF-β receptor kinases with porous polyethylene discs as the solid support.[33]

Toomik and Ek[40] 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 to give specific and efficient PKC substrates. Similar experiments led to optimized substrate sequences for the calcium-dependent kinase from maize seedlings.[49]

A different approach that proved to be very powerful for mapping antibody epitopes is a complete substitutional analysis[56] (for a review see ref. [18]). For example, substitutional analysis of a histone H3-derived peptide on cellulose membranes led to the discovery of DYRKtide, which is a very efficient peptide substrate for DYRK1a.[48] These strategies applied to peptide microarrays were also used to determine the substrate requirements of NEK6[22] and p60c-src.[29]

However, when a good database of substrates and nonsubstrates is available from peptide-array experiments, the generation of weight matrices is a valuable alternative for kinase specificity analysis. Weight matrices are a representation of the probability of each amino acid occurring at a certain position relative to the phospho-acceptor residue based on statistical evaluation of peptide microarray data. Phosphorylation by Abl of a library comprising 1433 randomly generated peptides resulted in a weight matrix that was successfully used to predict bona fide kinase substrates.[24]

Miscellaneous applications

A new high-throughout tool connecting solution-phase kinase-activity assays with immobilized format analysis via biotin-streptavidin interaction and Phosphor Imaging was introduced by Panse et al.[23] Following casein kinase II reactions in 384-well microtiter plates in the presence of [γ-^{32}P]ATP, aliquots of reaction solution were transferred to a streptavidin-coated membrane to create a peptide array composed of 720 different 13-mer peptides derived from human phosphorylation sites. Biotinylated substrate peptides were radioactively labeled due to the incorporated phosphate moiety and could be easily detected by Phosphor Imaging of the membrane after washing steps.

Zhu et al. described comprehensive analysis of yeast kinases using elastomer sheets with imprinted microwells mounted onto microscope slides.[57] The microwells were loaded with 17 different known substrate proteins by using the cross-linker 3-glycidoxypropyltrimethylsilane and incubated with 119 different yeast kinase–GST fusion proteins in the presence of radioisotope-labeled ATP. Subsequent to Phosphor Imaging, it could be demonstrated that this technology permits the identification of novel kinase activities. Generally, each protein microarray could be used for the analysis of kinase activities. Nevertheless, the correct alignment of identified phosphorylation events to a given peptide sequence is impaired by the number of potential phosphor-acceptor residues within a protein. Additionally, signal quantification is difficult due to possible multiple phosphorylations within one protein.

Another approach is the immobilization of kinase substrates on the bottom of a microtiter plate well, perfectly combining both formats. After incubation of the patterned peptide array inside the well with kinases and appropriately labeled phospho-specific antibodies, electrochemiluminescence detection can be used for signal readout.[58]

In a very similar approach, kinase substrate proteins were bound to the bottom of wells directly from cell lysates by using capture antibodies. The resulting protein microarray was analyzed quantitatively and kinetically with antibodies recognizing the tyrosine phosphorylation state of the receptors EGFR and ErbB2.[59] Additionally, it could be demonstrated that this approach is useful for estimating the effects of small-molecule inhibitors on ErbB signaling.[59]

An extension of the microarray approach is the determination of K_i values directly on microarrays. Houseman et al. demonstrated efficient concentration-dependent inhibition of c-Src activity for the c-Src inhibitors quercitin, tyrphostin, and PP1 by applying different kinase/inhibitor mixtures to a substrate-coated slide under a layer of mineral oil. Droplets formed due to the oil-layer reaction, thus allowing their spatial resolution.[31] Additionally, the authors were able to demonstrate that their peptide microarrays, on monolayers of alkanethiolates self-assembled on gold, are fully compatible with surface plasmon resonance spectroscopy[31] and MALDI mass spectrometry.[7]

Keywords: enzymes · kinases · microarrays · peptides · SPOT synthesis · substrate specificity

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