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REVIEW

Deciphering Enzyme Function Using Peptide Arrays

Alexandra Thiele · Gabriele I. Stangl · Mike Schutkowski

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Abstract Enzymes are key molecules in signal-transduction pathways. However, only a small fraction of more than 500 human kinases, 300 human proteases and 200 human phosphatases is characterised so far. Peptide microarray based technologies for extremely efficient profiling of enzyme substrate specificity emerged in the last years. This technology reduces set-up time for HTS assays and allows the identification of downstream targets. Moreover, peptide microarrays enable optimisation of enzyme substrates. Focus of this review is on assay principles for measuring activities of kinases, phosphatases or proteases and on substrate identification/optimisation for kinases. Additionally, several examples for reliable identification of substrates for lysine methyl-transferases, histone deacetylases and SUMO-transferases are given. Finally, use of high-density peptide microarrays for the simultaneous profiling of kinase activities in complex biological samples like cell lysates or lysates of complete organisms is described. All published examples of peptide arrays used for enzyme profiling are summarised comprehensively.

Keywords Substrate specificity · Kinase · Protease · Phosphatase · Peptide microarray · SPOT synthesis · Kinome profiling

Abbreviations

4Pip	Piperidine-4-carboxylic acid
Aa	Amino acid
Ab	Antibody
Abl	Abelson protein-tyrosine kinase
Abz	2-Aminobenzoic acid
Acc	4-Aminomethylcyclo-hexanecarboxylic acid
ACC	7-Amino-4-carbamoylmethyl coumarin
ACEi	Angiotensin converting enzyme inhibitor
Akt1	Protein kinase B isoform 1
Amb	4-Aminomethylbenzoic acid
ANPK	Androgen receptor interacting nuclear protein kinase
AP	Alkaline phosphatase
APC	Activated protein C
Aurora A	Breast tumour-amplified serine/threonine kinase
Aurora B	Aurora- and IPL1-like midbody-associated protein 1, protein serine/threonine kinase
Bcl2	Apoptosis regulator B-cell lymphoma 2

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A. Thiele · G. I. Stangl
Institute of Agricultural and Nutritional Sciences, Department of Human Nutrition, Martin-Luther-University Halle-Wittenberg, Von Danckelmann Platz 2, 06120 Halle, Germany

M. Schutkowski (✉)
Institute of Biochemistry and Biotechnology, Department of Enzymology, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Strasse 3, 06120 Halle, Germany
e-mail: mike.schutkowski@biochemtech.uni-halle.de

Bip	ß-(4-Biphenyl)alanine
BMK1	Big mitogen-activated protein kinase 1
Boc	tert. Butyl-oxy-carbonyl
BODIPY	(4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene) fluorophore
B-Raf	Tyrosine kinase-like kinase of the Raf family
Bth	ß-(3-Benzothienyl)alanine
Bz	Benzyl
CaMK2	Calmodulin dependent kinase 2
Cbz	Benzyl-oxy-carbonyl protecting group
C-CAM-L	Large isoform of cell–cell adhesion molecule
Cdc15	Cell devision control protein 15, yeast protein kinase
Cdk2, 5	Cyclin dependent kinase 2, 5
CDPK-1	Calcium dependent protein kinase
cGPKIa	cGMP dependent protein kinase Ia
Cha	3-Cyclohexyl-alanine
Chk1, 2	DNA damage checkpoint kinases 1 and 2
CK1, CK2	Casein kinase 1, 2
CMV	Cytomegalovirus
c-Raf	Tyrosine kinase-like kinase of the Raf family
c-Src	Sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue protein tyrosin kinase
Cy3	3H-indolium, 2-[3-[1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene]-1-propen-1-yl]-1-ethyl-3,3-dimethyl-5-sulfo-, inner salt
Cy5	3H-indolium, 2-[5-[1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene]-1,3-pentadien-1-yl]-1-ethyl-3,3-dimethyl-5-sulfo-, inner salt
DABCYL	4-(Dimethylaminoazo) benzene-4-carboxylic acid
DANSyl	Dimethylamino-naphthalen-sulphonic acid
Dap(Dnp)	2-Amino,3-(2′,4′-Dinitrophenylamino)-propionic acid
DAPK	Death-associated protein kinase
Dbf1-Mob1	Budding yeast protein kinase Dbf1 in complex with activator (Mps one binder 1)
Dbg	α,α-Dibutyl glycine
DegP	Degradation protein, serine protease HtrA
DEX	Dexamethasone
Dok6	Docking protein 6
DYRK1A, 2, 4	Dual-specificity tyrosine phosphorylation-regulated kinase isoform 1A, 2 and 4
EGFR	Epidermal growth factor receptor kinase
Elk1	Ets like gene 1
EphA4	Ephrin receptor tyrosine kinase isoform 4a
EphB2	Ephrin receptor tyrosine kinase isoform B2
Erk1, Erk2	Extracellular signal regulated kinases isoforms 1 and 2
Fes	Feline sarcoma (Snyder-Theilen) oncogene, protein-tyrosine kinase
FGFR-1	Fibroblast growth factor receptor 1
FITC	Fluorescein-thiocarbamoyl
Flt3	Fms-related tyrosine kinase 3
FRET	Forster resonance energy transfer
Fyn	Protein-tyrosine kinase of the Src family
GluR1	AMPAR glutamate receptor 1
Gsk3ß	Glycogen synthase kinase 3ß
H3, 4	Histone 3 and 4
HD1, 2	Histone deacetylase complexes 1 and 2 from *P. sativum*
Her2	Herstatin isoform 2

proto	Oncogenic receptor tyrosine kinase of the EGFR family
hK2	Glandular kallikrein 2
HRP	Horseradish peroxidase
InsR	Insulin receptor kinase
IR	Insulin receptor tyrosine kinase
Jak1 and 2	Janus protein-tyrosine kinases 1 and 2
JNK1and 3	c-Jun NH_2-terminal kinases 1 and 3
K^{Ac}	Side chain acetylated lysine
KDR	Kinase insert domain receptor
KDRI	Kinase insert domain receptor inhibitor
Kit	Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue, receptor tyrosine kinase
KPI-2	Kinase/phosphatase/ inhibitor 2
Lck	Lymphocyte cell-specific protein-tyrosine kinase
LMWPTP	Low molecular weight protein-tyrosine phosphatase
LPS	Lipopolysaccharid
Lyn	Protein-tyrosine kinase of the Src family
MAF	Mouse adult fibroblast
MALDI-TOF/MS	Matrix-assisted laser desorption ionisation—time of flight/mass spectrometry
MAP3K8	Mitogen-activated protein kinase 3 K8
MAPKAPK-2	MAP kinase activated protein kinase-2
MCA	7-Methoxycoumarin-4-yl)acetyl
MeAla	*N*-methyl-alanine
MEK1	MAP, Erk kinase 1
MELK	Maternal leucine zipper kinase
MePhe	*N*-methyl-phenylalanine
Met	Hepatocyte growth factor receptor, protein tyrosin kinase
MK2	Mitogen-activated protein kinase kinase 2
*m*LCEC	Mouse lung capillary endothel cells
MPK1, 2, 3, 6	Mitogen-activated protein kinases, isoforms 1,2,3, and 6
Nal	Naphthyl-alanine
NEK6	Never in mitosis gene A related kinase 6
NGF	Nerve growth factor
Nle	Norleucine
p70S6	Ribosomal protein S6 kinase
PABP1	Poly(A)-binding protein 1
PapA	Fimbrial major pilin protein A
PBMC	Peripheral blood mononuclear cell
PDE4B2, C2, A4, D5	Isoform B2, C2, A4 and D5 of phosphodiesterase 4
PDGFRß	Platelet-derived growth factor receptor kinase ß
PDGRF I-1	Platelet-derived growth factor receptor inhibitor 1
PDK1	3-Phosphoinositide dependent protein kinase 1
Pia	4-Piperidinylacetic acid
Pim1	Proviral integration site 1 kinase
Pin1	Peptidyl-prolyl-*cis/trans*-isomerase Pin1, protein interacting with NIMA 1
Pip	L-piperidine-2-carboxylic acid, pipecolic acid
PKA	Protein kinase A, cAMP-dependent protein kinase
PKCζ, PKCα, PKCδ	Protein kinase C isoforms
PKG	Protein kinase G
PKI	Protein kinase inhibitor
PknB	Receptor-like protein kinase from *Mycobacterium tuberculosis*
Plk1, Plk3, Plk4	Polo like kinase isoforms 1,3 and 4
PNA	Peptide nucleic acid
POD	Peroxidase
PP1, PP2, PP3, PP2A, PP2B	Protein phosphatase class 1,2,3, 2A and 2B
ppGalNACT	UDP-*N*-acetyl-ß-D-galactosamin:polypeptide

	N-acetylgalactosaminyl-transferase family
pSer	Phospho-serine
pThr	Phospho-threonine
PTPμ	Protein-tyrosine phosphatase μ
PTP1B	Protein-tyrosine phosphatase 1B
pTyr	Phospho-tyrosine
PVDF	Poly(vinylidene difluoride)
Pyk2	Proline-rich tyrosine kinase 2
QSY	Xanthylium, 9-[2-[[4-[carboxyl]-1-piperidinyl]-sulfonyl]phenyl]-3,6-bis(methylphenylamino)-, chloride
Rock2	Rho-associated kinase isoform 2
Rsk2	Ribosomal S6 kinase isoform 2
SAMDI	Self-assembled monolayers for matrix assisted laser desorption ionisation mass spectrometry
sec. ab	Secondary antibody
SGK1	Serum/glucocorticoid regulated kinase isoform 2
SHP1 and SHP2	SH2 domain containing phosphatase 1 and 2
SIR2	Silent information regulator 2
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SNAP25	Synaptosomal-associated protein 25
SPR	Surface plasmon resonance
SRPK4	SR protein kinase isoform 4
ß-Ala	3-Aminopropionic acid
ß-Gal	ß-Galactosidase
SSRP1	Structure-specific recognition protein 1
STAT5	Signal transducer and activator of transcription 5
Suc	Succinyl
TBB	4,5,6,7-Tetrabromobenzotriazole
TBK1	TANK-binding kinase isoform 1
tBuGly	α-tert. Butyl-glycine
tBuPhe	4-tert. Butyl-phenylalanine
TCPTP	T-cell protein-tyrosine phosphatase
Thi	ß-(2-Thienyl)-alanine
Thz	R-4-thiazolidine-carboxylic acid
Tic	1,2,3,4-Tetrahydro-isochinoline-3-carboxylic acid
Tie2	Tunica interna endothelial cell kinase 2
Tpk1	Tyrosine protein kinase 1
TrβI and TRβII	Transforming growth factor β receptor tyrosine kinases I and II
TSSK3	Testis specific serine/threonine kinase 3
uPA	Urinary plasminogen activator
VEGFR2	Vascular endothelial growth factor receptor kinase 2
Wt	Wildtype
Yes	Yamaguchi sarcoma viral oncoge, protein-tyrosine kinase
Z	Benzyl-oxy-carbonyl

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.

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 immobilisation of pre-synthesised peptide derivatives. In general, in situ synthesis has a number of advantages compared to immobilisation of pre-synthesised peptides. Normally, yields of peptides synthesised on surfaces are high and consistent over the entire support surface from one array region to another. It also permits combinatorial strategies for constructing large arrays of peptides in a few coupling steps. 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 optimised for high yields 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 synthesised). An interesting alternative circumventing this limitation is the use of photo-generated acids in combination with standard Boc-chemistry. [2–4].

Alternatively, the SPOT synthesis concept developed by Ronald Frank consists in the stepwise synthesis of peptides on planar supports, such as functionalised cellulose membranes, applying standard Fmoc-based peptide chemistry [5, 6]. 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 [7] 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 functionalised cellulose, aminated polypropylene [8] or aminopropylsilylated glass slides [9]. The areas contacted by the droplets represent individual micro-reactors 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 [8, 10–20].

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 (the high melting point of the solvent yields a toner-like powder) resulting in an activated amino acid solution during the laser-induced melting process [21–23]. Analogous ink-jet delivery of activated amino acids to appropriate functionalised surfaces, such as membranes, microscope slides [24] or spinning surfaces in a CD-format [25] 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 identical sequences are required, immobilisation of pre-synthesised peptides is more economical than in situ synthesis. Immobilisation is also the method of choice for longer peptide sequences, which normally have to be purified to obtain high quality products. Chemoselective immobilisation 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.

One intrinsic advantage of using chemoselective reactions via N-terminal reactivity tags 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 target peptide and the peptide synthesis protocol is modified by introduction of capping steps subsequent to each coupling reaction. Deposition of this mixture results in formation of a covalent bond exclusively between the full-length target peptide derivative and the surface. The chemically ‘inert’ truncated (and acetylated) 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 (see Fig. 1).

Different chemoselective reactions were applied 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, 26–30] or cysteinyl-residues [26, 31–34] 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 [35–40] and protease profiling [41]. It could be demonstrated that native chemical ligation, introduced by Dawson et al. [42] is well-suited for effective attachment of kinase substrate peptides containing an N-terminal cysteine residue to thioester modified glass slides [43–45]. A more sophisticated reaction for oriented immobilisation of peptide derivatives was introduced by Houseman et al. [46]. A Diels–Alder reaction between benzoquinone groups on self-assembled monolayers and cyclopentadiene-peptide conjugates led to efficient covalent attachment of kinase substrate peptides

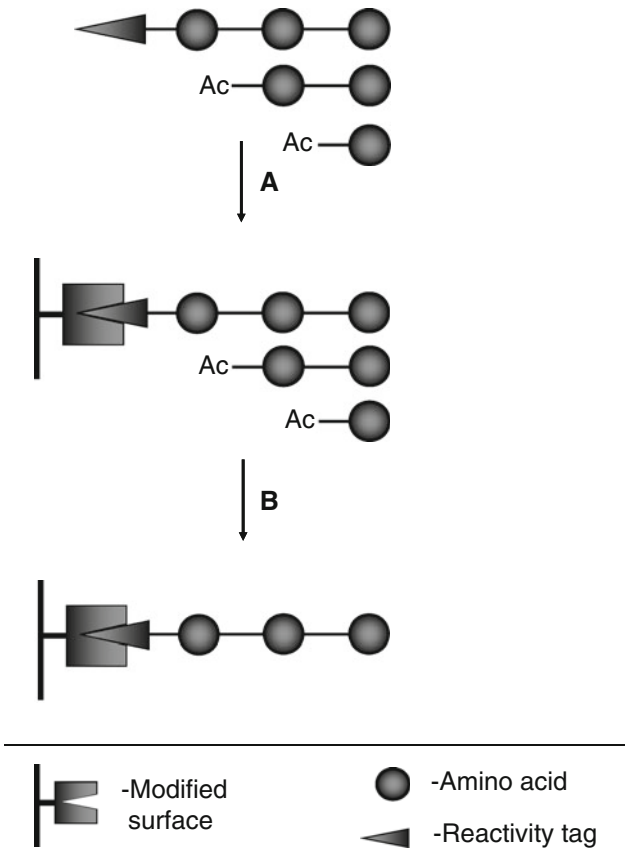

Fig. 1 Purification of peptides during chemoselective immobilisation. **a** deposition of peptides mixtures and chemoselective immobilisation of crude peptides which contain truncated sequences resulting from introduced acetylation steps subsequent to every coupling reaction. Truncated and acetylated peptides are non-reactive in contrast to the full-length target peptide equipped with the reactivity tag. **b** Washing steps subsequent to chemoselective immobilisation yield purified covalently immobilised target peptide

that were efficiently phosphorylated by c-Src kinase [46]. Formation of amide bonds via 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 [47]. Immobilisation of phosphopeptides containing alkyne-moieties onto sulfonylazide modified glass slides and subsequent dephosphorylation by protein-tyrosine phosphatase 1B could be demonstrated by Govindaraju et al. [48]. Regioselective immobilisation of poly(desoxythymidin)-modified kinase substrates onto differently coated glass slides was reported for PKA and c-Src [49]. Photocleavable acrylamide labelled cysteine-containing kinase substrates were incorporated into peptide-acrylamide 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 [50]. There are several additional chemistries used for the chemoselective immobilisation of peptides onto different surfaces (comprehensively reviewed in [19]) like formation of covalent bonds by reaction of salicylhydroxamic acids with 1,3-phenyldiboronic acid derivatives [51–53] or semicarbazides with aldehydes [54–60] or using thio-ene chemistry [61–64] but no applications for enzyme profiling have been described so far.

The surface-bound peptide's 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. In general, all linker molecules introduced to transform a given surface function into a functional group suitable for amino acid or peptide attachment can be considered as spacers. Such spacers can improve the efficiency of enzyme/substrate or antibody/peptide interactions on surfaces, as demonstrated with FLAG epitope peptides recognised by the monoclonal anti-FLAG M2 antibody [65]. 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 [26]. A similar linker structure was used to space apart Peptide Nucleic Acid (PNA) tags from potential protease substrates [66], protein serine/threonine kinase substrates [29, 30] or tyrosine kinase substrates [67]. 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 [35]. Moreover, insertion of hydrophilic dextran structures between the surface and the presented peptides was described as a prerequisite for efficient kinase substrate interaction [68]. Another alternative is the deposition of peptide-cellulose conjugates onto glass slides introduced as celluspots. Cellulose fragments could be considered as library of spacers and compatibility of celluspots with kinase assays was demonstrated [69, 70].

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 CK2 peptide substrates covalently attached to amino acid side chains of the albumin protein [71]. Alternatively, substrate peptides fused genetically to the C-terminus of human leptin were immobilised onto aldehyde modified glass slides [72]. Using this method, sensitivity was increased by three orders of magnitude compared to other microarray approaches with PKA and leptin-kemptide fusions. Lee et al. reported analysis of PKCβ substrate specificity by microarrays displaying peptide fusion to immobilised maltose binding protein [73]. Another method is the decoration of streptavidin with biotinylated peptides or phosphopeptides. Sun et al. were able to demonstrate

that biotinylated phosphopeptides immobilised onto streptavidin-coated glass surfaces could be efficiently dephosphorylated by phosphatases [74, 75] or isomerised by peptidyl-prolyl-*cis/trans*-isomerases [75]. Nevertheless, Kimura et al. could not find detectable phosphorylation of streptavidin-bound biotinylated PKA substrates by PKA using fluorescently labelled anti-phosphopeptide antibodies but were able to demonstrate phosphorylation of the same substrates if regioselectively immobilised via polythymine-desoxyribonucleoside tag onto aminosilane- or polycarbodiimide-coated glass surfaces [49].

Library Types for Profiling of Enzymatic Activities

A considerable number of different library types have been established 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 first type of 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 protein's sequence (see Fig. 2a) [30, 76–81]. Alternatively, libraries of peptides covering only the sequence around each potential phospho-acceptor residue or protease cleavage site have been used [82, 83]. The availability of high density peptide microarrays enabled the systematic extension of this approach in a 'proteomics-like manner', where addressing groups of

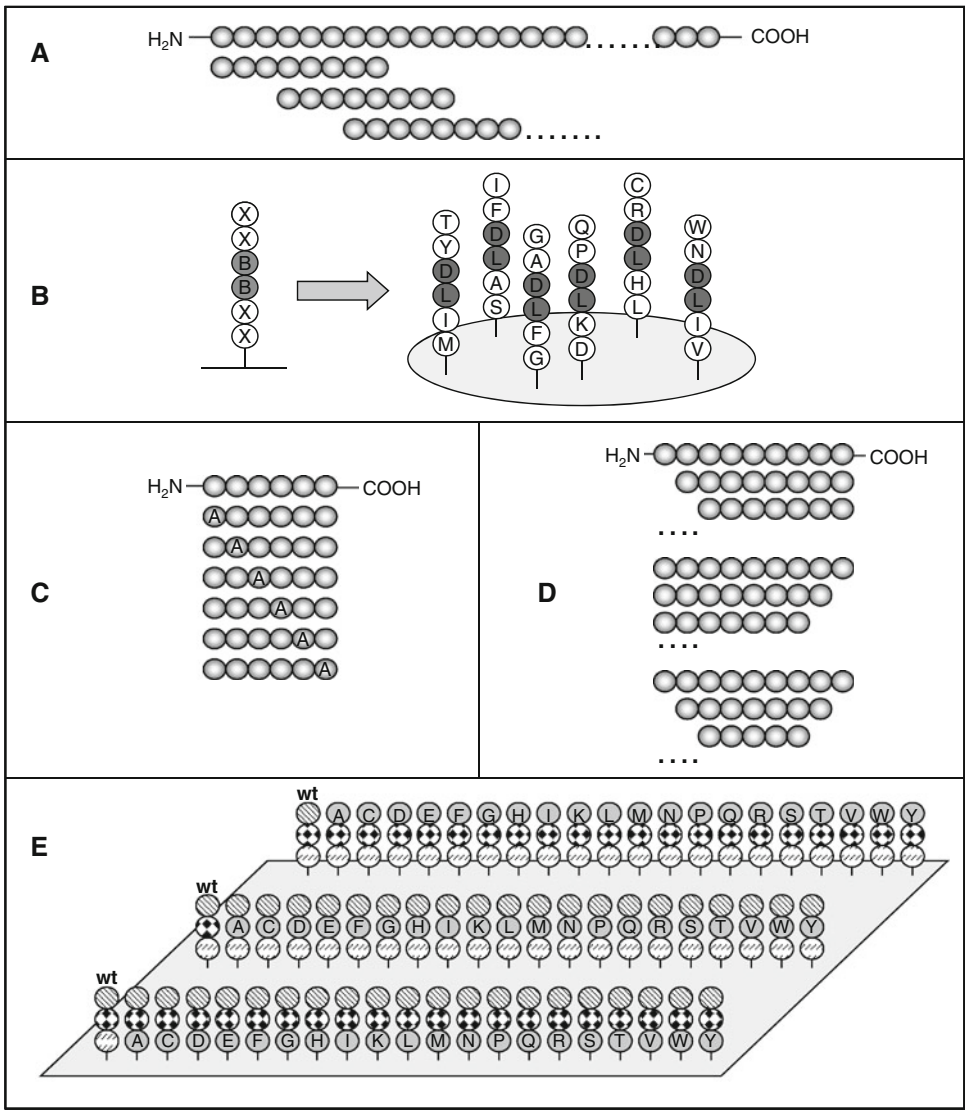

Fig. 2 Different library types. **a** Amino acid sequence of the protein under investigation is used to generate short linear overlapping 8-mer peptides shifted by three amino acids (peptide scan). **b** Peptide mixtures with defined positions B and randomised positions X. **c** Amino acid substitution scan (alanine scan) of a hexameric peptide. **d** Truncation library with N-terminal, C-terminal and bi-directional stepwise truncations. **e** Complete substitutional analysis of a 3-meric peptide

proteins comprising all human peptidyl-prolyl-*cis/trans*-isomerases in form of overlapping peptides resulting in more than 3,250 peptides or covering the complete proteome of human cytomegalovirus (17,181 overlapping peptides) immobilised onto one standard industry glass slide in duplicates. Incubation of these high content peptide microarrays with kinases and fluorescence scanning subsequent to treatment with phospho-specific dye yielded proteome-wide detection of phosphorylation sites [76]. In a similar approach, the sequences of experimentally identified phosphorylation sites taken from databases (i.e. Swissprot, [84] Phosphobase [85]) and the literature were comprehensively evaluated on peptide arrays [15, 27, 28, 30, 34, 38, 39, 86–96]. 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 which residue is phosphorylated when multiple phospho-acceptor sites occur in one individual peptide. When using overlapping 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 Fig. 3. Statistical analysis and alignment of the sequences proved to be useful in the case of collections of annotated phosphorylation sites [28]. Identification and alignment of key residues in the different substrate peptides allows a reliable assignment of the actual phospho-acceptor residue(s).

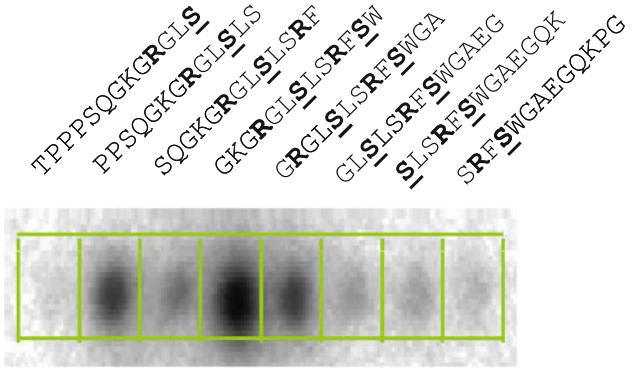

Fig. 3 Determination of phosphorylation sites using overlapping peptide scans. Section of the phosphorimage of a peptide microarray displaying a scan of overlapping peptides derived from myelin basic protein MBP (13-mers overlapping by 11 amino acids) after phosphorylation with protein kinase A in the presence of [γ-^{32}P]-ATP. Serine residues shown to be phosphorylated in the native MBP [223] are written in *bold* and *underlined*. The key residue arginine is shown in *bold*. The strongest signal is observed when both phosphorylation sites are in the central region of the substrate. When the respective phosphorylation site is positioned at one of the peptide termini phosphorylation is not effective

A further 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 lysine/arginine residues or acetylation of lysine residues, within the substrate sequences. This more adequately mimics the natural environment in which phosphorylation occurs, allowing the detection of peptide sequences becoming substrates only after an initial priming modification event. Such modifications can be introduced on-chip enzymatically after chemical synthesis of the unmodified peptides [30] or chemically either 'off-chip' using modified building blocks during the course of peptide synthesis [30, 74, 75] or 'on-chip' using chemoselective chemical reactions as demonstrated by selective acetylation of lysine side chains within microarray bound peptides [97] or selective methylations of lysine residues thereby creating 'chips from chips' [98–100].

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 synthesised 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 [101]. A very similar approach was used for more systematic mapping of protein/protein interactions [102, 103].

For de novo detection of kinase substrates, both the 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 randomised or degenerated positions [44, 68, 104–108]. Only one particular amino acid is introduced at the defined positions while a mixture of amino acids is introduced at the randomised positions, resulting in a sublibrary of different sequences within each single spot (see Fig. 2b). The number of individual sequences per spot depends on the number of randomised 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 randomised 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 guaranteed when using peptide microarrays with a low concentration of peptide per spot on planar surfaces [44].

The tremendous miniaturisation of peptide libraries feasible 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 [109–111]. Such randomly generated libraries for kinase substrate identification and kinase profiling have a defined phospho-acceptor residue and random sequences in the flanking areas [29]. 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 higher sequence diversity compared to knowledge-based libraries that are biased towards known kinase substrates. This can be an advantage in searching for selective substrates for closely related enzymes.

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 [28, 29, 105–108, 111, 112]. Alternatively, different library types based on single substrate sequences, such as alanine scans (see Fig. 2c), deletion libraries (see Fig. 2d) [44] and substitutional libraries (see Fig. 2e) [27, 86, 113–116] enable comprehensive characterisation of substrates on peptide arrays.

Assays and Detection

Enzymatic activity modifying peptides results either in addition of chemical moieties to peptides displayed on microarrays (kinases, acetyl-transferases, glycosyl-transferases and ADP-ribosyl-transferases, etc.) or in release of a part of the immobilised peptide derivative (proteases, phosphatases, histone deacetylases and lysine demethylases, etc.). Studies with kinases, phosphatases, proteases, (comprehensively reviewed in Table 1 in supplementary material), lysine methyl-transferases [117, 118], arginine methyl-transferases [119], histone deacetylases [120–123], peptidyl-prolyl-*cis/trans*-isomerases [75, 124–127], glycosyl-transferases [128–130], ADP-ribosyl-transferases [131, 132], hydrolases [133], esterases [134] and ubiquitin- or SUMO-ligases [135–137] have been described using peptide arrays on cellulose membranes or peptide microarrays on glass slides. This review will summarise applications of peptide (micro)arrays for kinase, phosphatase and protease research in more detail.

There are three 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. 4a). Subsequently, detection of incorporated radioactivity is achieved using either a phosphorimager [28–30] or X-ray films, or alternatively photographic emulsions that deposit silver grains directly onto the glass surface [71]. 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 [138, 139] and peptide microarrays [28–30, 91, 140, 141]. However, for reasons of operational safety, ease of handling and waste disposal, radioactive detection methods are increasingly avoided.

Alternatively, phosphopeptides can be detected using either phospho-specific antibodies (see Fig. 4b, c) or phospho-specific chelators (Fig. 4d, e) that are labelled with a detection moiety. The detection moiety can be a fluorescent label such as fluorescein [28, 31, 43] or an enzyme, for example horseradish peroxidase generating a chemiluminescent signal in combination with an appropriate substrate [101]. The detection moiety can be coupled either directly to the anti-phospho-amino acid antibody (see Fig. 4b) [15, 28, 44] or to a secondary antibody (see Fig. 4b) [46, 101]. Quality of anti-phospho-amino acid antibodies as detection tools was compared to the radioactive detection method generally recognised 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 2,234 monophosphorylated derivatives [28]. Monoclonal anti-phospho-tyrosine antibodies only showed reliable results with no detectable binding to non-phosphorylated amino acids. Additionally, such antibodies have a limited false-negative rate and low cross-reactivity to phospho-serine or phospho-threonine residues. 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 reported using peptide microarrays displaying more than 6,500 phospho-tyrosine peptides derived from human phosphorylation sites in triplicates [97]. In conclusion, the detection of phosphopeptides using antibodies seems to be limited to the detection of phospho-tyrosine residues, at least until suitable anti-phospho-serine and anti-phospho-threonine antibodies are available.

Additionally, phosphoamino acid chelators coupled to a detection moiety can be used (see Fig. 4d). Martin et al. described the Pro-Q diamond phospho-sensor dye which recognises phosphopeptides with remarkably little cross reactivity and a low false-negative rate [142]. It was demonstrated that this dye could be applied to kinase profiling [97, 143, 144] and phosphatase profiling [74, 75] using high density peptide microarrays. A very similar approach was reported using biotinylated zinc(II) chelate

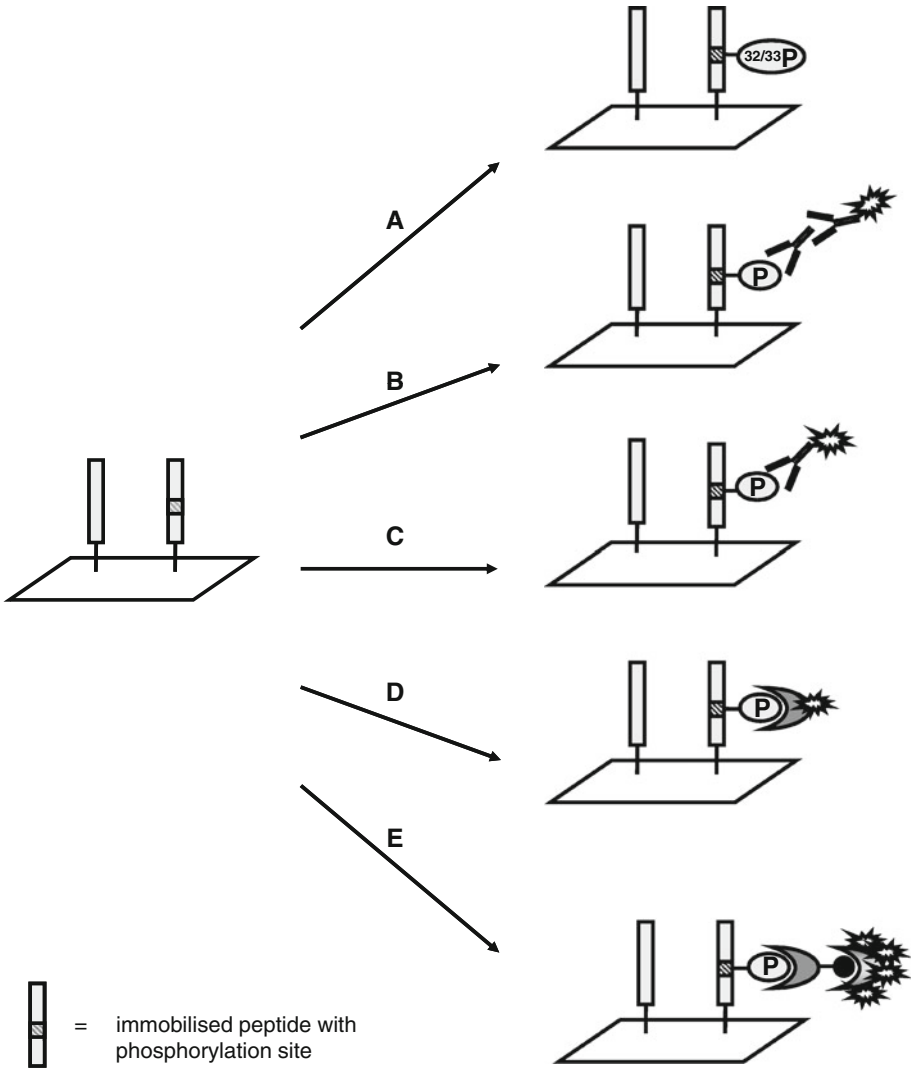

Fig. 4 Assay principles for the detection of peptide phosphorylation on peptide arrays. **a** Array is incubated with the kinase of interest in the presence of [γ-^{32}P or γ-^{33}P]-ATP and detection is performed by autoradiography. **b** Phosphorylation is measured with a generic anti-phospho-amino acid antibody in combination with a labelled secondary antibody. **c** Phosphorylation is detected with a directly fluorescently or chemoluminescently labelled anti-phospho-amino acid antibody. **d** Phosphopeptide detection using fluorescently labelled phosphoamino acid chelator. **e** Phosphopeptide detection using biotinylated phosphoamino acid chelator followed by fluorescently labelled streptavidin

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, see Fig. 4e) [34–37, 40].

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 [145]. Alternatively, Akita et al. used base-mediated 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 [146]. Enzymatic modification of the phosphate moiety could be performed using derivatives of ATP substituted at the γ-phosphate residue [147, 148]. Kerman and Kraatz used γ-thio-derivative of ATP to detect transfer of thio-phosphate to surface-bound peptides electrochemically using gold nanoparticles [149]. It could be demonstrated that electrochemical detection of kinase mediated transfer of modified phosphate residues is more efficient if electroactive adenosine-5′-[γ-ferrocene] triphosphate is used as co-substrate for protein kinase C [150]. ATP derivatives biotinylated at the γ-phosphate residue were applied to detect PKA or Src mediated generation of surface-bound biotinylated phosphopeptides using avidin-stabilised gold nanoparticles amplified by silver deposition [151, 152]. Use of biotinylated ATP derivatives in kinase assays seems to be an interesting alternative to existing technologies [153].

In principle, many other detection principles such as surface plasmon resonance and mass spectrometry are possible. Examples have been described for the detection of peptide phosphorylation on microarrays using resonance light scattering [153, 154], surface plasmon resonance [35, 36, 40, 46] and MALDI-TOF mass spectrometry [155, 156].

In general, 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. Surface-bound phosphopeptides could be generated either enzymatically [101, 157] or chemically [75, 101, 157–161]. Detection of phosphatase action on (micro)array bound phosphopeptides by signal decrease subsequent to treatment with anti-phosphotyrosine antibodies [101, 159, 160] or using phospho-specific dyes [75] was demonstrated.

Several, fundamentally differing 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. 5) or in still surface-bound peptide fragment (see Fig. 6). Several

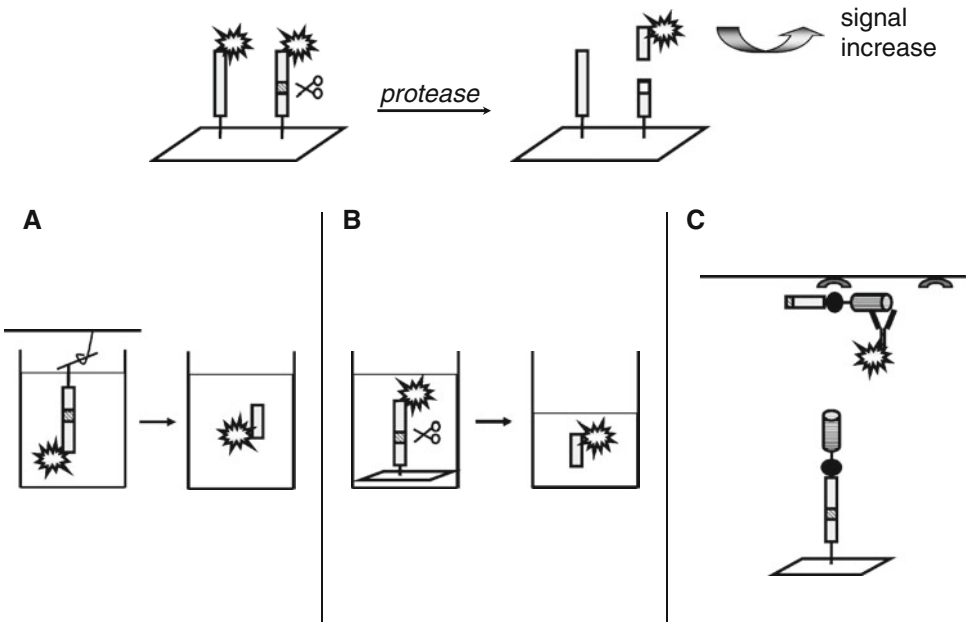

Fig. 5 Detection of proteolytic cleavage on peptide arrays reading released fragment. **a** N-terminally labelled immobilised peptides in the 96-well plate format, cleavage yields fluorescently labelled fragment released into the well of the microtiter plate; membrane disk with bound peptide fragment is release before fluorescence imaging [162]. **b** Alternative assay with N-terminally labelled immobilised peptides in the 96-well plate format, membrane disk with bound peptide or peptide fragment will stay in the well of the microtiter plate but released fragment is separated from membrane by transfer of aliquots of reaction solution from supernatant to other microtiter plate which yields fluorescence signals for cleaved peptides after fluorescence imaging [163, 164, 166, 167], and **c** indirect detection of peptide cleavage subsequent to electrotransfer of the released N-terminal peptide fragment containing antibody epitope [173]

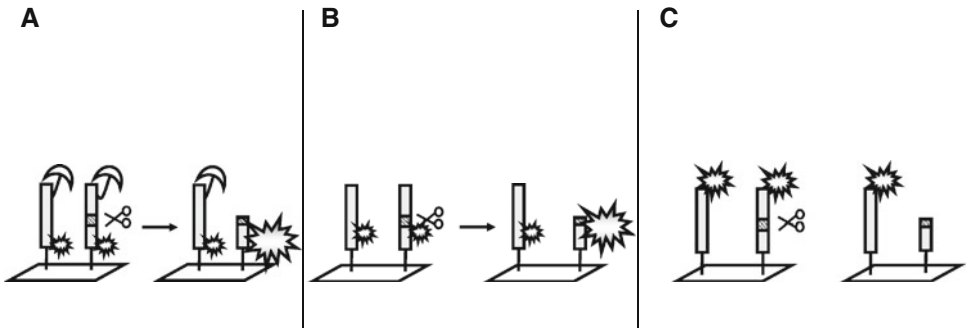

Fig. 6 Detection of proteolytic cleavage on peptide arrays reading surface-bound fragment. **a** Internally fluorescence-quenched peptides on arrays, subsequent to cleavage peptide fragment containing quenching moiety is washed away yielding fluorescence increase for cleaved peptides [66, 166, 171]. **b** Peptides having a fluorogenic group C-terminal to the scissile bond which increases fluorescence subsequent to cleavage [175, 181]. **c** Peptides with a fluorescence dye at the free terminus for array-based assays with decreasing fluorescence signal upon cleavage

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. 5a) [162]. The array comprised all 400 possible dipeptides derived from genetically encoded amino acids 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. This method was evaluated using chymotrypsin and papain [2]. To avoid the laborious pin attachment, a modified assay involves immersing substrate spots (N-terminally acylated with 2-amino-benzoic acid as fluorescence dye) in wells filled with the protease solution (Fig. 5b) [163–167]. At various times small aliquots are pipetted into fresh wells and cleavage is quantified using a fluorescence microtiter plate reader. This assay was employed to identify and characterise caspase-3 substrates using substitutional analyses of a known peptide substrate, a peptide scan, combinatorial libraries and randomly generated sets of peptides [166, 168, 169]. 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. 6a). Compartmentalisation 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 [170, 171] and subsequently employed to determine the substrate specificity of the integral membrane protease OmpT of *Escherichia coli* [171]. A similar assay principle was used for the fluorescence-based detection of botulinum neurotoxin A activity on peptide arrays [172]. 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. 5c) [4]. 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 [173]. 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 [174]. Peptide derivatives containing a substituted fluorogenic group C-terminal to the scissile bond are immobilised on glass slides resulting in peptide microarrays (see Fig. 6b) [5]. In a proof-of-concept study, it was demonstrated that the protease trypsin cleaved the amino acyl-fluorophore bond [133]. 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 immobilisation [175]. Collet et al. used fluorous-coated glass slides for immobilisation of substituted peptide-fluorophore conjugates and demonstrated fingerprinting of protease plasmin, chymotrypsin, trypsin, thrombin and granzyme B using resulting peptide microarrays [176]. Peptides with a fluorescence dye at the free terminus are applied for array-based protease assays with a decreasing signal upon cleavage (see Fig. 6c) [6].

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 [177]. Lysyl endopeptidase treatment released an environmentally sensitive fluorophore resulting in a blue shift of the emission maximum from 540 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 [178, 179]. The peptide nucleic acid tag encodes the structure of the attached peptide derivative and, therefore, allows spatially addressed deconvolution after hybridization to Affymetrix GenFlex oligonucleotide microarray. This approach was extended to the profiling of enzymatic activities of proteases [66, 174, 180, 181] and kinases [67, 145] using PNA/DNA-encoding. The use of oligonucleotides and in particular peptide nucleic acids (PNAs) to encode libraries was reviewed comprehensively [182].

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 (see Fig. 2b) [68,

107, 108, 112, 138], PKG [106, 108, 138] and the budding yeast kinase CDC15 [107]. This approach was also successful using peptide microarrays for p60c-src [44]. A randomly generated library of 1,433 tyrosine-containing single peptides on a peptide microarray was used to identify new substrates for c-Abl [29].

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 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 used to overcome this problem.

In cases where 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-meric peptides generated from the sequence around each tyrosine residue in PKC [82]. Similary, all tyrosine-containing 13-meric 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 [80, 184]. Decapeptide sequences derived from the cytoplasmic domains of C-CAM revealed a single specific phosphorylation site for PKC [83]. Overlapping peptide scans were used to determine PKA phosphorylation sites in myelin basic protein (Fig. 2), the autophosphorylation sites as well as sites for CK2-mediated phosphorylation in the tyrosine kinase Tie2 [30], CK2-mediated phosphorylation sites in SSRP1 [78], CaMK2-mediated phosphorylation of Ets-2 [79], autophosphorylation sites in Eph receptor tyrosine kinase [80], Gsk-3β phosphorylation sites in p65 NFkappaB [77] and phosphorylation sites for several human kinases in all human cyclophilins, FK506 binding proteins and parvulins [76].

Even though initial proof-of-concept experiments with 18 cellulose membrane bound peptides derived from protein sequences phosphorylated by PKC in vivo [87] and later studies [184] were successful, the full power of the knowledge-based approach only emerged when applied to high density, high content peptide microarrays. Peptide microarrays displaying the sequences of more than 700 human annotated phosphorylation sites revealed peptide substrates for NEK6 [27], Abl [28], PKA/CK2/Gsk3 [30] and 13-meric peptide substrates specific for splice variants of dual-specificity tyrosine phosphorylation-regulated kinase 4 [141]. More advanced libraries were used for CK2 (11,096 peptides from cytoplasmic domains of human membrane proteins and 2,304 human annotated phosphorylation sites) [28, 90], protein kinase complex Dbf2-Mob1 [90], PDK1 (1,394 peptides derived from the activation loops of human kinases) [30] and CK2 or Gsk3 (17,181 peptides representing overlapping peptides covering complete proteome of cytomegalovirus) [76]. 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 experiments [27, 30].

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) [185]. Moreover, microarrays displaying more than 1,000 peptides derived from human phosphorylation sites enabled the identification of novel substrates for receptor tyrosine kinase Flt3 [69], mitogen-activated protein kinase kinase 8 [186] and transmembrane serine/threonine kinase KPI [187]. In a similar way, peptide microarrays displaying 144 different phosphopeptides derived from known human phosphorylation sites were used to fingerprint the substrate specificity of different but highly homologous phosphatases (PTP1B, TCPTP, ΔSHP1, ΔSHP2, SHP1, SHP2, LMWPTP) [74].

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 Gsk3 as second kinase with a library of 694 annotated human phosphorylation site peptides where all corresponding CK2 monophosphorylated derivatives were generated by incorporating phospho-amino acid building blocks during synthesis [30]. Similar approach was described by Coba et al. using protein kinases Gsk3, CaMKII, Erk2, ROCK-II, Fes and JNK3 [188].

Some evidence has accumulated 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* [91]. 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 characterise the substrate

specificity of mitogen-activated protein kinases from tomato [87], big mitogen-activated protein kinase 1 from mice [89], protein kinase 7 from *P.falciparum* [189], proline-directed kinase PknB from *S.aureus* [190], kinase activities in sugar-stimulated and sorbitol treated *A.thaliana* cells [88, 191], tyrosine kinase activities in jasmonate and/or salicylate treated *A.thaliana* cells [192], kinase activities in mouse osteoblast cell lines [193] and tyrosine kinase activities in zebrafish embryos [38, 39].

Substrate Optimisation

From the beginning, the use of peptide arrays on cellulose membranes in kinase research focused on kinase substrate optimisation in terms of substrate efficiency and selectivity. Using cAMP- and cGMP-dependent protein kinases (PKA and PKG) as model enzymes, Tegge et al. [108] applied peptide arrays on cellulose membranes to identify substrates from combinatorial libraries with the format Ac-XXXO_1O_2XXX where X represents mixtures of all 20 proteinogenic amino acids, and positions O_1 and O_2 represent individual amino acids defined individually for one single spot but varying between different spots in the library. Incorporation of all the 20 naturally encoded amino acids at these two defined positions will result in $20 \times 20 = 400$ spots each displaying a peptide mixture composed of 20^6 members. After a first screening round of this initial library, the best two amino acids at positions 1 and 2 are retained throughout the optimisation cycles and two new positions are defined. This procedure is iteratively repeated 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 [194]. Extending this approach to 12- and 14-meric peptides yielded substrates highly specific for PKG [105]. 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 [112, 106]. Additionally, similar combinatorial libraries led to the deconvolution of substrate sequences for PKA and type I and II TGF-α receptor kinases using porous polyethylene discs as the solid support [68].

Toomik and Ek [86] used the SPOT technology to synthesise an optimisation library for PKC substrates, with the flanking residues of a known substrate substituted by different amino acids, leading to specific and efficient PKC substrates. Similar experiments led to optimised substrate sequences for the calcium-dependent kinase from maize seedlings [114].

A different approach that proved to be very powerful for mapping antibody epitopes is a complete substitutional analysis (see Fig. 2e) [195]. 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 [113]. These strategies applied to peptide microarrays were also carried out to determine the substrate requirements of NEK6 [27] and p60c-src [44]. Moreover, iterative substitutional analyses led to optimised substrates for murine Plk4 [196] and EphA4 receptor tyrosine kinase [183].

However, when a good database of substrates and non-substrates 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 of a library comprising 1,433 randomly generated peptides by Abl resulted in a weight matrix that was successfully used to predict bona fide kinase substrates [29].

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 [38, 39]. 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 tumours [197, 198]. Additionally, synergistic effects on multiple carcinoma cell lines could be demonstrated for the kinase inhibitors lapatinib and pazopanib using a similar approach [70]. 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 tumour 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 kinase reactivity patterns are different for chondrosarcoma cell cultures and colorectal carcinoma cell lines [199]. 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 [200]. Recently, Schmerwitz et al. identified cyclin-dependent kinase 9 as target for flavopiridol in TNF-α treated primary human umbilical vein endothelial cells [201].

Additionally, this approach was used to analyse the effects of treatment with glucocorticoid [94, 95], cyclooxygenase-2 inhibitor Celecoxib [202], spongistatin [203], lipopolysaccharide [92], ACE inhibitor Ramipril [204], EGFR tyrosine kinase inhibitors Gefitinib and Erlotinib [205] and anti-CD45RB antibody [206] on kinase reactivity profiles of the respective cell lysates. Bowick et al. used kinase reactivity profiling to characterise the host cell response to lethal arenavirus infections [207]. In zebrafish embryos, effects of morpholino-mediated protein knock down as well as changes in the embryonic development on phospho-tyrosine signalling were analysed using tyrosine kinase reactivity profiling in combination with peptide microarrays displaying several hundred tyrosine phosphorylation site-derived peptides [38, 39]. Finally, enzyme reactivity profiles could be indicative for the status of a cell. Sikkema et al. identified characteristic tyrosine kinase reactivity profiles in 29 paediatric brain tumours [208], Ter Elst et al. detected unique tyrosine kinase reactivity profiles in leukaemia samples [209], Bratland et al. found increased phosphorylation levels revealing EGFR and Erb2 mediated signalling in androgen-sensitive prostate carcinoma cells [198], Jinnin et al. analysed tyrosine phosphorylation patterns in lysates from hemangioma endothelial cells [210] and van Baal et al. were able to detect three unique kinase reactivity profiles in biopsies of 27 Barett's oesophagus patients as compared to normal squamous oesophagus [93]. Additionally, Folkvord et al. could demonstrate that phosphoproteome profiling of 67 patients might predict tumour response to preoperative chemoradiotherapy in locally advanced rectal cancer [211]. Using peptide microarrays, Taher et al. were able to detect differences in tyrosine phosphorylation in B-cells from patients with Systemic Lupus Erythematosus compared with matched controls [212] and Vivanco et al. identified a critical role for PTEN in EGFR signal termination using peptide microarrays displaying more than 140 human tyrosine phosphorylation site peptides [213]. Milani reported use of peptide microarrays displaying more than 1,000 human phosphorylation sites for profiling the phosphoproteome of MC3T3 pre-osteoblast mouse cell line seeded on polystyrene surfaces [193]. Thus, it might be anticipated that extension of this methodology to more features on the microarrays and to other enzymatic activities, like lysine side chain acetylation [214] and methylation [99], will generate novel biomarkers for cancer and autoimmune diseases in the near future.

Miscellaneous

A new high throughput tool connecting solution-phase kinase activity assays with immobilised format analysis via biotin–streptavidin interaction and phosphorimaging was introduced by Panse et al. [28]. Following casein kinase 2 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 labelled due to the incorporated phosphate moiety and could be easily detected by phosphorimaging of the membrane after washing steps [28]. A similar approach was used in combination with positional scanning libraries for deciphering substrate specificity of plant protein kinases [215], *S. cerevisiae* kinases Ime2 and Cdk1 [216], and eight different human protein serine/threonine kinases [217]. Prisic et al. extracted 336 phosphorylation sites within *M. tuberculosis* proteins and profiled substrate specificity of nine different *M. tuberculosis* serine/threonine kinases. Subsequent to treatment with the kinase in the presence of ^{33}P-ATP and binding of the 13-meric-biotinylated peptides to streptavidin-coated plates incorporated radioisotopically labelled phosphate moieties were measured by scintillation counting [218]. Zhu et al. described comprehensive analysis of yeast kinases on elastomer sheets with imprinted microwells mounted onto microscope slides [219]. The microwells were loaded with 17 different known substrate proteins using the cross-linker 3-glycidoxypropyltrimethylsilane and incubated with 119 different yeast kinase-GST fusion proteins in the presence of radioisotope labelled ATP. Subsequent to phosphorimaging it could be demonstrated that this technology permits the identification of novel kinase activities. In general, 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.

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 c-Src inhibitors quercetin, 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, allowing their spatial resolution [46]. 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. This MALDI MS imaging was extended to six different single kinases or two phosphatases, kinase mixtures and K562 cell lysates in the absence and presence of inhibitors in combination with microfluidic devices [156].

Alternatively, binding measurements on peptide arrays could be used to determine the substrate specificity of enzymes indirectly. This was demonstrated for peptidyl-prolyl-*cis/trans*-isomerases like trigger factor [126, 127] or Pin1 [124, 125]. Recently, an unbiased screening strategy was developed to identify potential substrates for human sirtuin 3 [123]. Cellulose membranes displaying either peptides from mitochondrial proteins or substrate analogues containing thioxylated trifluoroacetyl-lysine residues were used to screen for high affinity binding to the active site of sirtuin 3 [220]. Results were used to predict mitochondrial substrates for this human enzyme [123]. A related approach is the use of phosphatase variants which are catalytically inactive but still bind to the target substrate peptides (so-called substrate-trapping mutants). Espaniel et al. used GST fused to substrate-trapping mutant (D181A) of protein-tyrosine phosphatase 1B, which was radioisotopically labelled by treatment with PKA, for binding studies on phosphopeptide-displaying cellulose membranes [101, 160]. Pasquali et al. were able to identify the substrates within the intracellular domain of human GHR by incubation of phosphopeptides immobilised on cellulose membranes with substrate-trapping mutants of PTP-H1, PTP-1B, TC-PTP and SAP-1. Autoradiography enabled detection of bond, radioisotopically labelled GST-fusion proteins [161]. Substrate specificity of five different phosphatases was determined indirectly by treatment of phosphopeptide microarray with appropriate, fluorescently labelled substrate-trapping mutants [221]. A high content peptide microarray displaying more than 6,000 phosphotyrosine-containing human peptides was generated to identify novel substrates for PTP-1B [222] and tumour suppressor density-enhanced phosphatase-1 [157]. The GST-fusions of the respective substrate-trapping mutants were incubated with the microarray followed by treatment with fluorescently labelled anti-GST antibody.

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