Automated Synthesis

High-Content Peptide Microarrays for Deciphering Kinase Specificity and Biology

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Enzymatic protein phosphorylation is one of the key regulatory events in biologically important processes such as cell-cycle regulation, metabolism, and signal transduction. Protein kinases are thought to comprise the largest enzyme group in the human genome and are important targets in drug discovery. Recent estimates suggest that there are approximately 500 genes encoding protein kinases.[1] However, only a small fraction of these enzymes have thus far been characterized and little is understood concerning the physiological functions, substrate specificity, and downstream targets of the vast majority of these enzymes. A key step in the biological characterization of kinases and in their use as drug targets is the identification of their cellular interaction partners and the identification of suitable substrates for kinase-inhibitor development.

For a minority of protein kinases, for which cellular substrates have been identified, a knowledge-based approach can be employed to extract information concerning the localization of phosphorylation sites and to deduce peptidic substrates. Alternatively, combinatorial peptide libraries have been used for kinase profiling.[2] A macroarray-based approach used collections of peptides arranged in a spatially addressable format and allowed the direct identification of peptidic kinase substrates.[3] The use of peptide macroarrays produced by direct synthesis on cellulose membranes (SPOT technology)[4] is, however, limited by the number of peptide substrates on the macroarray, the high amount of enzyme needed, and the potential unspecific binding of the kinase and adenosine triphosphate (ATP) to the porous cellulose membrane. These limitations can be overcome by testing peptide microarrays produced either by direct synthesis or by immobilization of peptides on nonporous surfaces, preferentially modified glass or silicon wafers.[5,6] Recently, proof-of-concept experiments for kinase profiling by using peptide microarrays have been reported.[5,7] Here, we describe the generation of different types of high-density peptide microarrays, which display up to 6912 covalently attached peptides on one microscope slide, as a robust tool for the characterization of kinase specificity and biology.

Peptides for display on the microarray were synthesized by SPOT technology, released from the cellulose membrane,[8] and immobilized on glass surfaces. We used the aminooxyacetyl moiety as a reactivity tag coupled to the N terminus of the peptides to allow chemoselective attachment on aldehyde-modified glass surfaces. The hydrophilic linker 1-amino-4,7,10-trioxa-13-tridecanamine succinimic acid[9] was inserted between the reactivity tag and the peptide sequence to make the peptides more accessible for the enzymes. We used a noncontact printing device for the deposition of the peptides onto the functionalized microarray surfaces (for details, see the Supporting Information).

We extracted 710 human phosphorylation sites as 13-meric peptides from public databases (Swiss-Prot rel. 4.0 and Phosphobase version 2.0)[10,11] and prepared an appropriate microarray (for the entire list of peptide sequences, see the Supporting Information). Figure 1 summarizes the results of an experiment with cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA). The sequences of the top 20 substrates that were detected enabled us to deduce a consensus sequence matching the known RRXS consensus for PKA[2,11] (X = any amino acid). Other kinases have been

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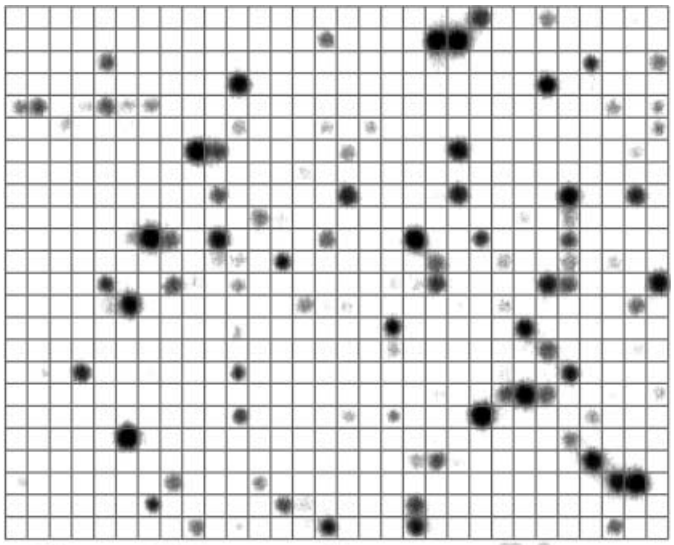


S22:	PGLGRKL**S**DFGQE	N06:	EEGTFRSSIRRL**S**
V29:	EPLERRL**S**LVPDS	U28:	RSAIRRA**S**TIEMP
K07:	LSAFRRT**S**LAGGG	I26:	FMRLRRL**S**TKYRT
R24:	TASTRKV**S**LAPQA	K10:	GQKFARKS**T**RRSI
T06:	ANRERRP**S**YLPTP	M25:	NIHLEKKYVRRD**S**
G09:	RFIGRRQ**S**LIEDA	O24:	RAGKRRP**S**RLVAL
U27:	NPLMRRN**S**VTPLA	X19:	NQNSRRP**S**RATWL
K19:	RSGSRRG**S**FDATG	G22:	RGAPPRRS**S**IRNA
B20:	NYLRRRL**S**DSNFM	D12:	QKRREILSRRP**S**Y
B21:	RLQRRRG**S**SIPQF	O18:	LLNKRRG**S**VPILR
N30:	KNGCRRG**S**SLGQI	Q26:	RKSKRRN**S**EFEIF

Figure 1. Kinase substrate peptide microarray displaying 710 peptides derived from annotated human phosphorylation sites after incubation with PKA and ^{32}P-ATP. The lower panel shows the coordinates and peptide sequences corresponding to the most intensive signals in decreasing order. The substrate consensus is highlighted in gray.

successfully profiled on the same type of peptide microarray (data not shown and ref. [12]). The proteins related to the substrate peptides identified on the array represent potential downstream targets of the profiled kinase. Another important aspect for kinase regulation is their autophosphorylation. We used overlapping peptide sequences of kinases displayed on microarrays to study autophosphorylation events (see the TIE2 kinase example in the Supporting Information).

Kinase substrate specificities may also depend on prior sequential posttranslational modifications steps, such as phosphorylation by another kinase, an event designated as priming phosphorylation.[13] This cellularly important regulatory mechanism could be simulated and elucidated with casein kinase 2 (CK2) and the β isoform of glycogen synthase kinase 3 (GSK3β) on a microarray. Separate incubation of the microarray with either CK2 or GSK3β and ^{32}P-ATP resulted in a distinct phosphorylation pattern for each kinase (Figure 2). We "preincubated" the microarray with CK2 and nonradioactive ATP to demonstrate that "priming" the peptide substrate with CK2 leads to novel specificities for GSK3. This first experimental step led to nonvisible phosphorylation of the microarray by CK2 kinase. The right panel in Figure 2 displays subsequent phosphorylation of the "prephosphorylated" microarray with GSK3β, now in the presence of radioactive ^{32}P-ATP. Comparison of the experiments shown in Figure 2 reveals peptide D15 as being sensitive to priming phosphorylation. Peptide D15 (QASS^{653}PQSS^{657}DVEDE) is derived from human liver glycogen synthase, a known protein substrate for GSK3β. According to the literature, serine residue 653 is a GSK3β substrate only after phosphorylation of serine 657.[13]

Additionally, we designed a phosphopeptide microarray for analyzing priming phosphorylation in a more general approach. We immobilized 694 annotated human phosphorylation-site peptides together with all possible corresponding monophosphorylated peptides (resulting in 2234 phosphopeptides) on a microarray, thereby simulating all pre-monophosphorylation events in one experiment. After incubation with CK2 (data not shown) we identified two peptides (Figure 3) with improved substrate properties if the tyrosine residue in the +3 position is phosphorylated. The kinetic constants for CK2 were determined in a solution-phase assay and showed a clear improvement in the value of the Michaelis constant K_m for FDNNEEESSYS-pY-E over the unprimed peptide (maximum rate $V_{max} = 15.7\ \mu mol\,min^{-1}\,mg^{-1}$ and $K_m = 0.3$ mM for primed peptide versus $V_{max} = 10.2\ \mu mol\,min^{-1}\,mg^{-1}$ and $K_m = 1.4$ mM for nonphosphorylated peptide). In contrast, no substrate properties are detectable for RPRGQRDSSYYWE. However, priming phosphorylation generates a substrate (RPRGQRDSSY-pY-WE: $V_{max} = 4.4\ \mu mol\,min^{-1}\,mg^{-1}$ and $K_m = 0.4$ mM).

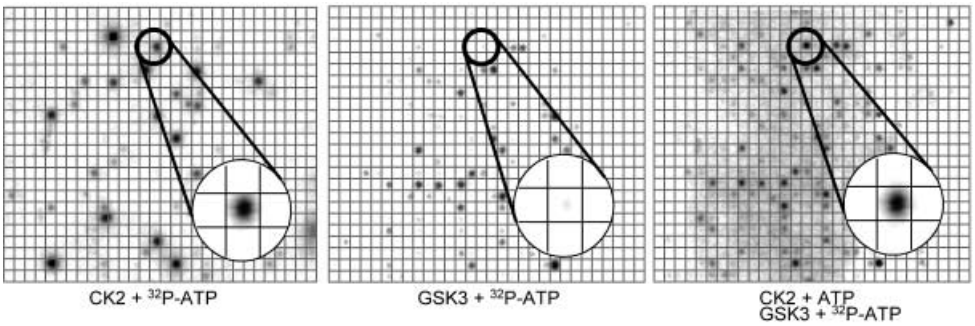


Figure 2. Priming phosphorylation of kinase substrates. Image of a microarray identical to that in Figure 1 after incubation with ^{32}P-ATP and CK2 (left panel) or GSK3 (middle panel), respectively. In the right panel, the array was "preincubated" with CK2 and nonradioactive ATP. This was followed by an incubation with GSK3 in the presence of ^{32}P-ATP. The signal for peptide QASSPQSSDVEDE derived from glycogen synthase is enlarged in each panel.

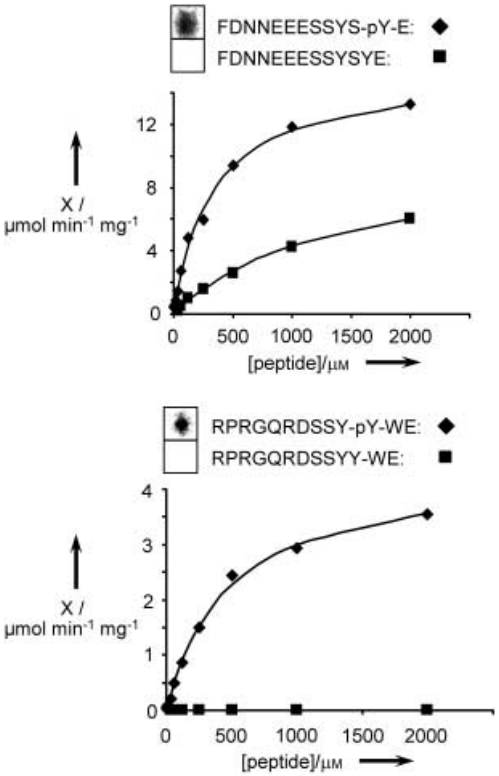
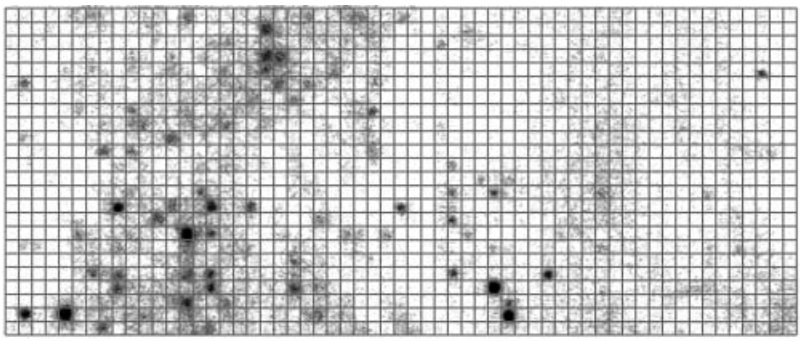


Figure 3. Conversion (*X*) of the CK2-mediated phosphorylation of peptides FDNNEEESSYSE and FDNNEEESSYS-pY-E (top) or RPRGQRDSSYYWE and RPRGQRDSSY-pY-WE (bottom, pY = phosphotyrosine). The signals from the corresponding microarray experiments are shown on top of the diagrams and labeled with the appropriate sequence.



1. FAKTFVGTPYYMS	NEK2	6. LAKSFGSPNRAYT	CDK7
2. LARAFGVPVRTYT	cdc2-related	7. LARAFGVPLRTYT	CDK3
3. DGLLKMLSLVLMH	FRAP	8. RRNTFIGTPYWMA	HPK/GCK like
4. QNMFRNFSFMNPR	PKC-theta	9. MRTLCGTPTYLAP	Chk2
5. RRDSFIGTPYWMA	SLK	10. NSFVGTRSYMSPE	MKK 1

Figure 4. Detection of potential downstream targets for PDK1. A peptide microarray, containing 1394 peptides derived from activation loops[23] of human kinases, after incubation with PDK1 in the presence of ^{32}P-ATP and 25 mM PIFtide peptide.[20] The ten best substrate peptides of PDK1 and the corresponding kinases are listed in the lower panel according to their signal intensity.

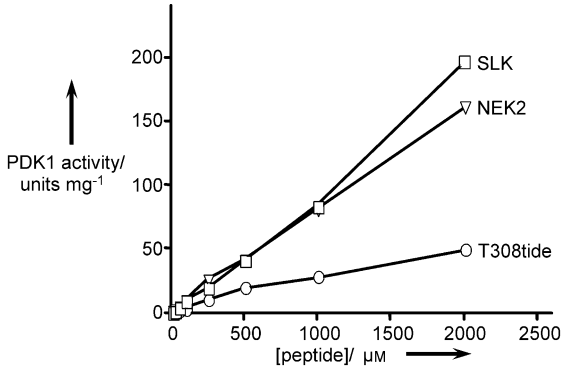


Figure 5. Kinetic analysis of the phosphorylation of two microarray-derived substrates (see Figure 4, sequences 1 and 5) and the known peptidic PDK1 substrate T308tide (KTFCGTPEYLAPEVRR) in solution.

The activity of kinases is often regulated by phosphorylation events, mediated by other kinases, in the activation-loop region. To analyze this type of important regulatory event in kinase biology, we prepared a peptide microarray containing 1394 peptides derived from activation loops of human kinases to search for novel downstream targets of 3-phosphoinositide-dependent protein kinase (PDK1; Figure 4). PDK1 is responsible for activation-loop phosphorylation and, thus, activation of certain members of the AGC subfamily of protein kinases that includes protein kinase B (PKB),[14] p70 S6 kinase,[15] serum- and glucocorticoid-induced kinase,[16] protein kinase C isoforms,[17] p21-activated kinase,[18] and PKA.[19] PDK1 itself needs an autophosphorylated activation loop together with an occupied exosite (PIF binding pocket) for full activity.[20] This activation of PDK1 can be simulated by the addition of an oligopeptide (PIFtide) to the assay solution.[21] No incorporated phosphate could be detected after incubation of the activation-loop microarray with only PDK1 and ^{32}P-ATP. However, we found several substrates after the addition of PIFtide to the PDK1 assay solution (Figure 4). Interestingly, many of the detected sequences are not related to the known in vivo substrates. The identified NEK2- and SLK-derived peptides represent substrates for PDK1 with improved kinetic parameters compared to the known substrate derived from the activation loop of PKB (T308tide; Figure 5). Moreover, in contrast to T308tide, our novel peptidic substrates are not equipped with the APE motif on the C-terminal side of the phosphorylation site. This APE motif was thought to be an essential recognition motif for efficient binding to PDK1.[22] Incorporation of such a moiety into the NEK2- or SLK-derived sequences did not improve the kinetic parameters, a result indicating that, at least for these substrates, the APE motif is not a key determinant for recognition by PDK1 (data not shown). Activation-loop-derived peptides of MKK1 and MKK6 as well as the full-length proteins were also found to be phosphorylated by PDK1 in vitro. The phosphorylation is stimulated by PIFtide (Figure 6). There is no physiological evidence that NEK2, SLK, MKK1, or MKK6 are in vivo substrates for PDK1 but the results presented in Figures 4–6 demonstrate that peptide microarrays are useful mining tools that enable the rapid identification of in vitro substrates for protein kinases.

In summary, we have introduced high-density peptide microarrays as a highly efficient and robust tool in kinase research. The preparation of the arrays was enabled by the ultra-high-throughput automated synthesis of peptides by the

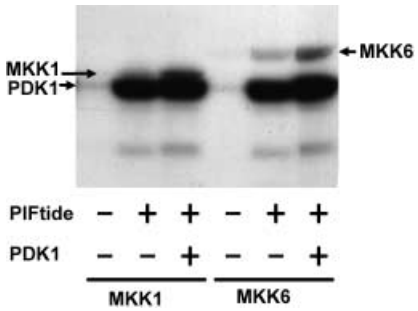


Figure 6. Autoradiography of a polyacrylamide gel electrophoresis (PAGE) gel after phosphorylation of proteins MKK1 and MKK6 with PDK1 in the presence and absence of PIFtide.

SPOT technology, which allows the synthesis of thousands of peptides in parallel on a microscale level. It was shown that the flat glass surface is ideally suited for profiling kinases. This approach potentially allows the highly parallel and efficient study of all human, viral, bacterial, and fungal kinases as potential drug targets.

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