Identification of Targets of the Protein Kinase A using engine protein arrays

Introduction

Enzymes that catalyze the transfer of the gamma phosphate group from ATP to the side-chain hydroxy (OH) group of amino acids like serine, threonine or tyrosine are called protein kinases. About 2% of all genes in the human genome code for kinases. The approximately 518 kinases can phosphorylate nearly 30% of all proteins in a cell.

In this study we focus on cAMP-dependent protein kinase A (PKA) aiming to identify targets and thereby getting some more insight into the target preference of the kinase. PKA was discovered in 1968, belongs to the serine/threonine protein kinases and is a well-studied model kinase including structural features, target recognition, and kinetics [1]. As a holoenzyme, the kinase consists of two catalytic and regulatory subunits. These regulatory subunits prevent unregulated activity of the kinase, and the catalytic subunit transfers the phosphate from the donor to the acceptor. PKA is ubiquitously expressed and in human cells activated via the cyclic adenosine monophosphate (cAMP) pathway. For the analysis presented, the catalytic subunit was used. The catalytic PKA subunits targets the consensus sequence (R/K)-(R/K)-X-(S/T) [2]. In addition, the catalytic subunits can also be inhibited by pseudo-substrates and other inhibitors like H89.

Protein arrays allow several thousand analytes to be analyzed in one experiment under identical settings [3]. Therefore, phosphorylation of a protein array bears the opportunity to screen a large number of possible substrates for protein kinases and analyze them in depth as described above for the targeted consensus sequence of the kinase.

engine protein arrays

engine offers a selection of protein arrays for the analysis of auto-antibody profiles of complex sera, cross-reactivity of monoclonal antibodies and other types of protein-protein interactions. On each of these arrays, several thousand different proteins are expressed and immobilized directly on polyvinylidene fluoride (PVDF) membranes (Figure 1). Therefore, post-translational modifications of expressed proteins, like citrullination or phosphorylation, can be introduced on the array. Clones are arranged in multiple copies in distinct patterns to avoid false positive results (see Figure 2).

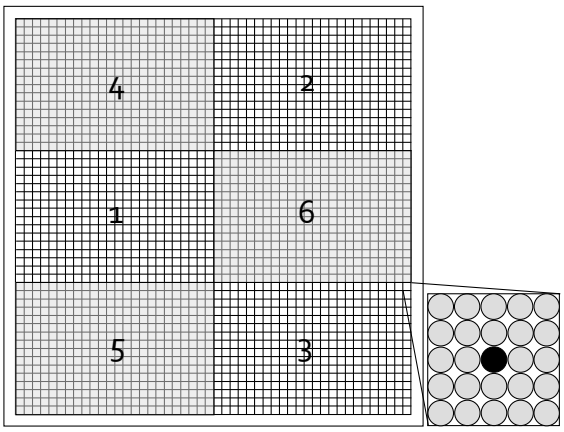

Figure 1: Schematic view of an engine protein array, printed in 5x5 pattern. Numbers 1-6 indicate the fields, clones from 384 well plates are spotted into. Clones are spotted there in distinct patterns (see Figure 2) in duplicate, serving as a build-in positive control, since spots are only considered positive, if both spots can be detected. Pattern 1 is spotted in fields 1-6 from the first six 384 well plates. Pattern 2 starts from plate 7 in field 1 and so on. By doing so, up to > 27.000 clones can be placed on the array in duplicate. Zoomed out part shows one block of the array. In 5x5 patterns, one block consists of up to 12 clones in duplicate and one central guiding dot.

cDNA collection

To construct the hEX1 expression library for engine protein arrays, cDNA was prepared from either human fetal brain, using poly(A)+ RNA by oligo(dT) priming. The products were directionally cloned into modified vectors for

IPTG-inducible expression of His_6-tagged fusion proteins and transformed into either *E. coli* SCS1 cells [4]. After selection of the desired clones by checking for expression, they were rearrayed into 384-well microtiter plates and *N*-terminally sequenced for verification of the cloned gene.

Spotting of expression libraries for engine protein arrays

To produce engine protein arrays, PVDF membranes are activated with ethanol, washed twice with water, and incubated with LB-medium. Then, the membranes are positioned on pre-soaked Whatman paper and transferred to the spotting robot. There, the clones arranged in 384-well microtiter plates, are spotted onto the membrane using a spotting device with 384 pins.

For hEXselect protein arrays used in this setup, clones are spotted in a defined duplicate pattern within spotting squares that consist of up to 12 duplicate clones and one central reference spot per square (5x5 pattern, Figure 2). They contain >24,000 cDNA clones, covering over 7,000 unique human proteins.

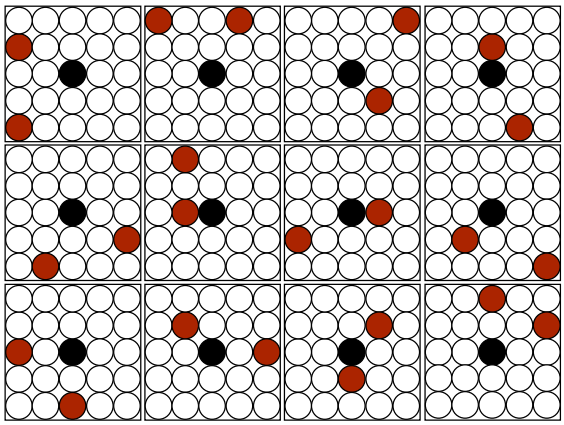

Figure 2: Patterns 1-12 (from left to right, highlighted in red) in which clones are spotted in duplicate in a 5x5 pattern on engine protein arrays. The black center spot marks the guiding dot for the block. All patterns are spotted in each block. For better visibility, they are shown here in separate blocks and highlighted in red.

Protein library expression and protein array production

For expression, bacterial clones on the membrane are incubated overnight on 2×YT-agar plates containing 2 % glucose and 100 µg/ml ampicillin at 37 °C and on the next day, induced for protein expression for 3 h at 37 °C on agar plates containing 1 mM IPTG. These protein filters are denatured on pre-soaked blotting paper in denaturation buffer* for 10 min, neutralized for 2× 5 min with neutralization buffer* and incubated for 15 min in NaPi buffer*. The proteins on the arrays are verified for expression by detecting the His_6-Tag using an anti-His antibody RGS·His (Qiagen), 1 array per spotting run.

*Denaturation buffer – 0.5 M NaOH, 1.5 M NaCl
*Neutralization buffer – 1 M Tris–HCl, pH 7.5, 1.5 M NaCl
*NaPi buffer – 0.05 M di-sodium dihydrogen-phosphate, pH 7.2

Materials and Methods

Phosphorylation of protein arrays

Two hEXselect protein arrays were first activated with ethanol and washed with demineralized water (2x) before excess colony material was wiped off the surface of the array. All following steps (except for substrate incubation) were carried out under agitation of the arrays using a "Rocky" shaker. After equilibrating the arrays for 5 minutes in PKA-buffer*, they were incubated for 30 minutes at 30°C in 30 ml PKA-buffer with 6,8 µg PKA (Proteinkinase.de, PK-PKA-CA050) and 10 µl 100 mM ATP (Roche, #11140965001) or 30 ml plain PKA-buffer. In the next step, they were washed 10 min with washing buffer (40 ml) and then twice with 40 ml washing buffer + 1 % SDS to remove the PKA from its targets, to avoid the detection of autophosphorylation of the kinase and pseudo-substrates.

*PKA-buffer – 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7,2

Protein array probing

After washing once with water, the protein arrays were incubated in blocking solution* for 2 hours followed by overnight incubation with 5 µl anti-Phospho-PKA substrate (Cell Signaling Technologies, #9624) in 40 ml blocking solution* at room temperature. This solution was discarded the next day, and the arrays were washed with wash buffer* + 1 % SDS at RT for 3 x 10 minutes. The secondary antibody (sheep anti-rabbit IgG alkaline phosphatase (AP) coupled, Seramun, A-016-1-AP) was diluted 1:5000 in blocking

solution* and 50 ml per array were used for incubation of 2 hours at RT. The arrays were then washed with washing buffer* + 1 % SDS (2 x 10 min), TBS* (2 x 10 min), and APE buffer* (1 x 10 min). 1 ml of Attophos substrate (Roche, 11681982001) was diluted in 20 ml of APE buffer*. 10 ml of this solution was used for one array and after incubation for 5 min, the array was placed protein side down on the surface of a fluorescence scanner (Storm 860, Molecular Dynamics) and fluorescence was exited at 450 nm.

*Washing buffer – Candor wash buffer
*Blocking solution – Candor BSA block
*TBS – 10 mM Tris-Cl, pH 7,5, 150 mM NaCl
*APE buffer - 1 mM $MgCl_2$, 100 mM Tris-HCl, pH 9,5

Analysis of images

The *.gel images were acquired by scanning with a Storm 860 fluorescence scanner from Molecular Dynamics, using an optical resolution of 100 pixel per cm. Analysis of the images was performed using VisualPattern20, an analysis program, specifically designed to analyze engine protein arrays. While analyzing the arrays, hits are categorized by the user into 3 intensities. 1 – low intensity, 2 – medium intensity and 3 – high intensity. Hits were only considered positive if both spots were clearly visible.

Analysis of sequences

For further analysis of the PKA target sequences, the existing sequences of positive clones were screened for the known consensus sequence. In the next step, the surrounding amino acids were analyzed, and an amino acid profile was created.

Results

The control array, incubated with plain PKA-buffer showed 3 positive interactions exhibiting a very low intensity with anti-Phospho-PKA substrate (Figure 3) and the anti-rabbit IgG antibody.

For the hEXselect array that underwent protein phosphorylation by PKA, 305 interactions with varying fluorescence intensities could be detected (Figure 4). Hits of intensities 2 and 3 can easily be reviewed in the picture.

Of the 305 positive interactions, 121 clones were categorized into intensity 1, 115 clones into intensity 2 and 69 clones into the highest intensity of 3.

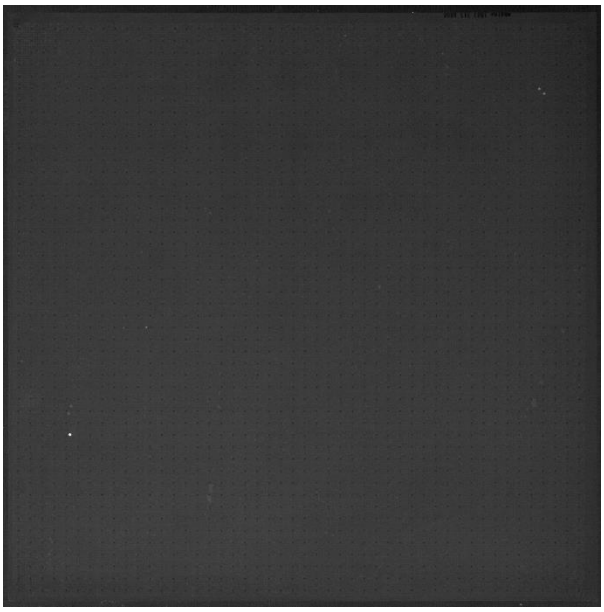

Figure 3: Control - engine hEXselect protein array incubated for 30 min at 30°C in PKA-buffer without PKA and ATP and probed with anti-Phospho-PKA-substrate and anti-rabbit IgG-AP-antibody. Positive hits were detected using Attophos substrate a fluorescence scanner (Storm 860). Three low signals could be detected.

For all clones partial sequences exist about the inserts and thus which potential regions of the human proteins are expressed. These regions of the protein sequences were searched for the presence of the PKA target sequence (R/K - R/K - X - S/T). A total of 249 potential PKA target sequences were identified in 202 of the 305 clones. Among the identified clones are known PKA targets such as ribosomal protein L15 (RPL15) or CLIP2 and new potential target proteins such as PPIA and SDK2. Of some proteins, such as NCOR2, several clones with different inserts of the protein could be identified.

PKA also binds to so-called pseudosubstrates. To identify potential pseudosubstrates, the sequence (R/K - R/K - X - X) was searched for in all available protein sequences, whereby the second X (P position) must not be a serine or threonine. A total of 103 proteins contained such a sequence. A more detailed analysis showed that at the P -1 position there is a slight preference for glycine, but all amino acids are represented.

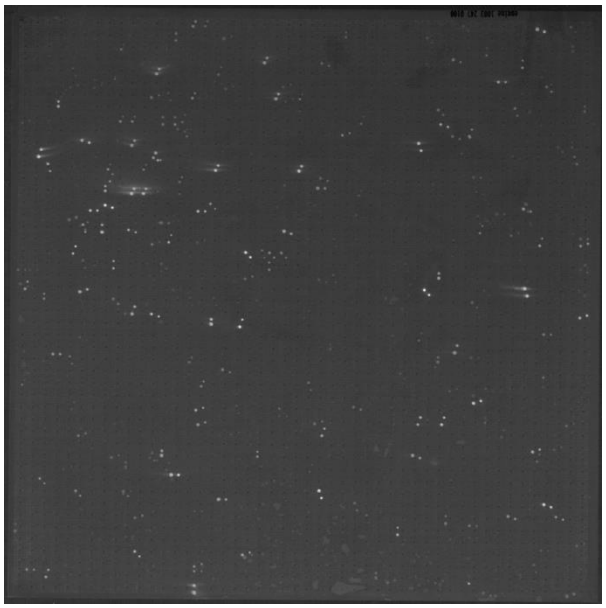

Figure 4: Phosphorylation - engine hEXselect protein array incubated for 30 min at 30°C in PKA-buffer containing PKA and ATP. After phosphorylation of the proteins, the array was probed with anti-Phospho-PKA-substrate and anti-rabbit IgG-AP-antibody. Positive hits were detected using Attophos substrate a fluorescence scanner (Storm 860). A total of 305 clones with varying interaction-intensities were detected.

Conclusion

Our data show that protein arrays are excellent for identifying potential targets for kinases. Due to the large number of proteins, information on the specificity of the kinases can be obtained in addition to the identification of potential target proteins. The relatively simple experimental setup allows the use of protein arrays for screening reaction conditions such as different pH values or co-factors.

Acknowledgements

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