Unravelling antibody specificity employing multiple complementary approaches

Sandra Mükusch [2,3] ,Matthias Knape [1], Harald Seitz [2] and Friedrich W. Herberg [1*]
1 Kassel University, Dept. Of Biochemistry, Heinrich Plett Straße 40, 34132 Kassel
2 Fraunhofer Institute for Celltherapy and Immunology Branch Bioanalytics and Bioprocesses Potsdam-Golm, Am Mühlenberg 13, 14476 Potsdam, Germany
3 Free University of Berlin, Institute for Chemistry and Biochemistry, Takustraße 3, 14195 Berlin, Germany

INTRODUCTION

Phosphospecific antibodies are widely used for the analysis of phosphorylation patterns mediated by protein kinases. For this, a large number of commercially available antibodies against phospho-Tyr and phospho-Ser/Thr exist. In general, these antibodies are generated using synthetic (phospho) peptides that bear a canonical consensus sequence for a respective protein kinase. The functionality, specificity and quality of antibodies differ between techniques used for production, batches and need to be evaluated individually. In this study we analyzed the interaction of substrate peptides of cAMP-dependent protein kinase (PKA), where the canonical recognition site
(Arg- Arg -X-pSer) was systematically modified with a widely-used PKA-substrate antibody. Three different techniques were used:

1. Peptide Microarrays
2. Bead-Based Assay (Luminex Technology, BioPlex3D)
3. Surface Plasmon Resonance (SPR, Biacore)

MATERIAL AND METHODS

Three different methods were used to unravel the specificity of the most common PKA-Substrate AB. Therefore, the canonical recognition site (RRxpS) was systematically altered within peptide sequences (Table 2, Discussion). In Table 1 the general set up of the experiment for the individual platform is described. For Peptide Microarrays and the Bead-Based System peptides are used as ligands and were incubated with the PKA-Substrate AB. The detection was performed with fluorescent antibodies. Within the SPR experiments the PKA-Substrate AB (ligand) was captured and peptides (analyte) were injected in different concentrations to determine the affinity and kinetic values.

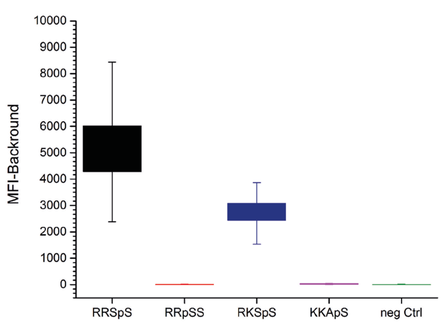
Table 1 Assay Setup

Platform	Ligand	Ligand concentration	Analyte	Detection
Peptid Microarray	Peptide	50 pg per Spot	PKA-Substrate AB	Fluorescent AB
Bead-Based Assay	Peptide	5 µg per $1 * 10^6$ Beads	PKA-Substrate AB	
SPR	PKA-Substrate AB	(> 97 % active surface)	Peptide (2 µM-16 nM)	SPR

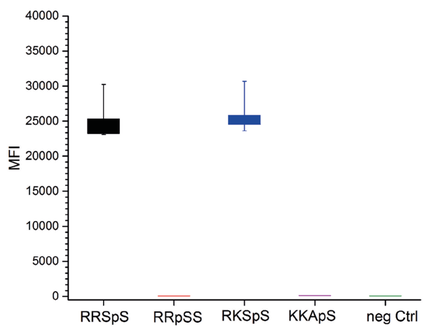
RESULTS

For the following figure a subset of the peptide library is depicted to illustrate the different binding capacities of the PKA-substrate AB depending on the changes of the canonical recognition site on three independent platforms.

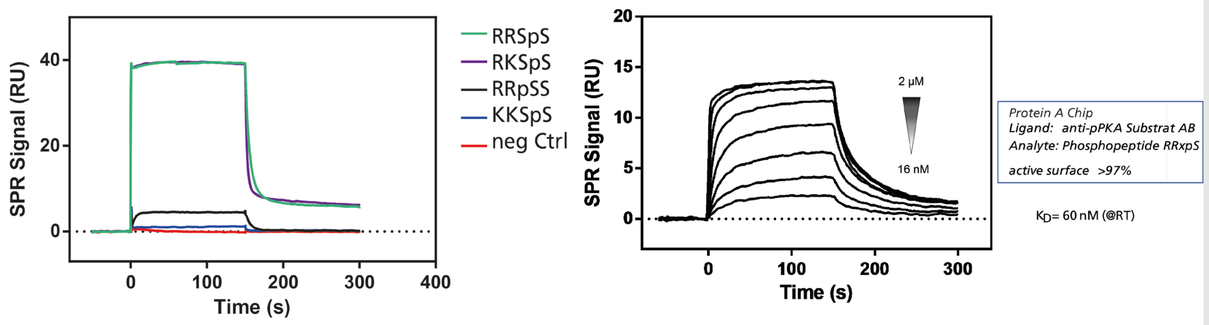
A Peptide Microarrays



B Bead-based Assay (Luminex technology)



C Surface Plasmon Resonance (SPR, Biacore)



Analyt concentration: 80 µM

Figure 1 Platform comparison to unravel the binding capacities of PKA-Subtrate AB

Peptide Microarray

Detected:

- Canonical recognition site (RRx**pS**)
- P-2 Lys (RKS**pS**)

Not Detected:

- non-phosphorylated Peptide
- P-3 and P-2 Lys (KKA**pS**)
- Phosphorylation next to Arg (RR**pS**S)

Bead-Based Assay

Detected:

- Canonical recoginition site (RRxpS)
- P-2 Lys Peptide (RKspS)

Not Detected:

- non-phosphorylated Peptide
- P-3 and P-2 Lys (KKA**pS**)
- Phosphorylation next to Arg (RR**pS**S)

Surface Plasmon Resonance

Detected:

- Canonical recoginition site (RRx**pS**)
- P-2 Lys Peptide (RKS**pS**)

Weak Binding:

- P-3 and P-2 Lys (KKS**pS**)
- Phosphorylation next to Arg (RR**pS**S)

Not Detected:

- non-phosphorylated Peptide

Binding Kinetics

- K_D can be evaluated for peptides e.g. RRx**pS** K_D: 60 nM (@RT)

Table 2 Detection of the modified canonical recognition site (Arg- Arg -X-pSer) for the PKA-substrate AB
(n.d.: not detected, weak detection (✓), good detection ✓

Motiv	Peptid Mikroarray	Bead-Based Assay	SPR
RRSS	n.d.	n.d.	n.d.
RRAS	n.d.	n.d.	n.d.
KKSS	n.d.	n.d.	no data
KKAS	n.d.	n.d.	n.d.
RRApS	✓	✓	✓
RRSpS	✓	✓	✓
RRpSS	n.d.	n.d.	(✓)
RASpS	✓	✓	✓
ARSpS	n.d.	n.d.	n.d.
RKSpS	✓	✓	✓
KRSpS	n.d.	n.d.	(✓)
KKApS	n.d.	n.d.	(✓)
KKpSS	n.d.	n.d.	n.d.
KKSpS	n.d.	n.d.	n.d.
pSRRPS	n.d.	n.d.	no data
pSRRPpS	No data	✓	✓

CONCLUSION

Three methods were used for an in-depth analysis of the phospho-site specific PKA-Substrate antibody. Most of the following results were achieved cross-plattform (Table 2).
Yet a a few important conclusions can be drawn from the binding behaviour of the PKA-Substrate antibody, since all peptide variations are rather good PKA-substrates *in vitro* and *in vivo*. Therefor, relative quantification of phosphosites using anti-phospho substrate antibodies is likely prone to errors.

- Non-phosphorylated peptides are not recognized by the antibody
- P-3 Arg is a major determinant for the antibody binding
- P-3 Lys has weak antibody binding properties (SPR)
- P-2 Arg or Lys enhances the binding affinity
- P-1 Ala better than Ser
- Phosphorylation at P-1 is not or only weakly detectable (SPR)

In-depth analysis of antibody recognition sites is for example useful in terms of finding new substrates for kinases with a variety of different peptide sequences on multiplexing platforms to prevent false positive/negative results. Every method has it´s advantages and disadvantages like multiplexing capabilities, binding kinetics, handling, time, costs. The method of choice has to be evaluated individually.

ACKNOWLEDGEMENTS

This work was supported by the BMBF Grantnumber 0316177

Bundesministerium für Bildung und Forschung

Contact: Dr. Harald Seitz| Fraunhofer-Institute for Celltherapy and Immunology | Branch Bioanalytics and Bioprozesses | Dept. Bioanalytics & Biosensoric | AG Biomarker-Validation & Assay Development | Am Mühlenberg 13 | 14476 Potsdam-Golm | Telephone +49 331 58187-208| E-mail harald.seitz@izi-bb.fraunhofer.de | www.izi.fraunhofer.de