Application Note # 125

Studying the binding kinetics of single chain antibodies of certain specificities by MP-SPR

Introduction

PKCε (protein kinase Cε) plays essential roles in a variety of signaling systems and dysregulation of PKCε is involved in several life-threatening diseases such as cancer, type II diabetes and Alzheimer's disease. Therefore, specific activators and inhibitors of PKCε are promising as research tools and as future drugs. Llama single chain antibodies (VHHs) are a new category of monoclonal antibodies that can specifically activate or inhibit human PKCε. A recent publication reports a kinetic analysis of these VHHs with PKCε by surface plasmon resonance (SPR) [1].

These VHHs were also tested in kinase activity assays to determine their kinetics of activation or inhibition of PKCε. In addition, these VHHs have *in vivo* PKCε activity, since they have different effect on the translocation of PKCε in HeLa cells.

The results presented here show that the kinetics measured by SPR, activation/inhibition and translocation of PKCε have a good correlation.

Materials and methods

The affinity measurements were performed with purified monoclonal VHH antibodies [2] using BioNavis SPR Navi™ 200 multiparametric surface plasmon rensonance instrument (MP-SPR) in traditional SPR fixed angle mode. Carboxymethylated dextran hydrogel was self-synthesized according to the BioNavis protocol. The PKCε immobilization to the hydrogel was done by reverse amino-coupling using EDC/NHS after amino-modification of the hydrogel. All the experiments were carried out using serial dilutions of VHHs at 21 °C and at a flow rate of 20 ml/min. Analysis of the SPR results was performed with TraceDrawer 1.3 for BioNavis. The sensograms were fitted with either first or second order Langmuir binding models.

Kinase activity assays were carried out as described before [2]. For translocation studies HeLa cells were transfected either with control, A10-, or G8-plasmids. After phorbol 12-myristate 13-acetate (PMA) stimulation translocation was quantified by measuring the relative fluorescence in the cytoplasm for 30 min.

More detailed description of the methods applied in the study can be found from the original publication [1].

Results and discussion

Affinity measurements with SPR showed, that when PKCε was carboxyl-coupled to the surface of the flow cell, VHH binding to PKCε was detected (Figs. 1 and 2). This was not the case after amino-coupling of the protein suggesting, that the binding of VHHs to PKCε is conformation dependent.

The binding of VHHs A10, C1, D1 and E6 to PKCε was best fitted with second order Langmuir binding models. This suggests that PKCε was present in two or more different conformations on the surface, and that binding of the VHHs to two of these conformations, having the strongest affinities, could be detected. The G8 data was fitted with a first order Langmuir model.

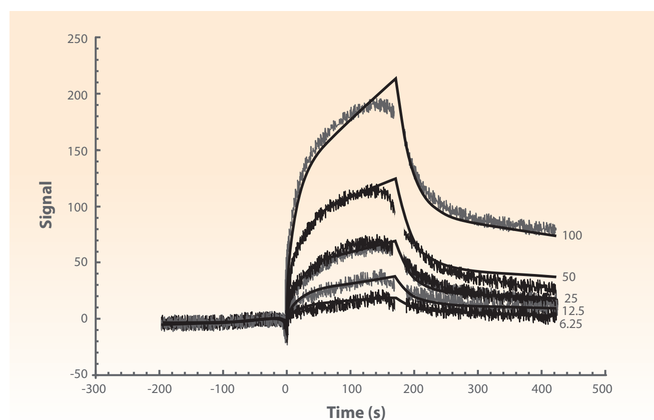

Figure 1. Example SPR sensograms and fit for second-order Langmuir binding model for VHHs D1. Used concentrations of each VHH (g/ml) are indicated.

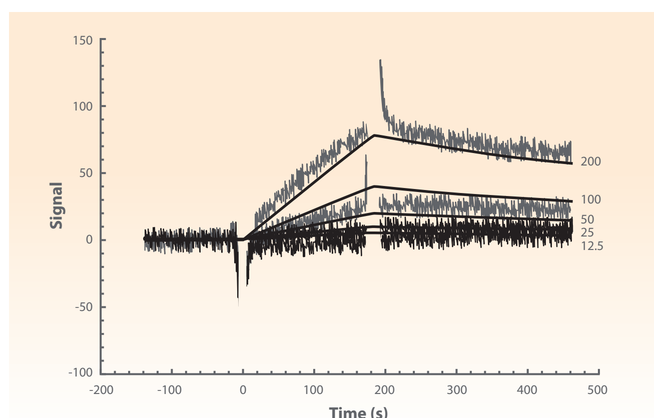

Figure 2. Example SPR sensograms and fit a first-order Langmuir binding model for VHH G8. Used concentrations VHH (g/ml) are indicated.

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Out of the activators (A10, C1 and D1), C1 had the highest affinity for PKCε, and D1 was ranked as a second. A10 had the lowest affinity of the three activators (Fig. 1, Table 1). In kinase activity assays, C1 caused the greatest increase in PKCε activity, followed by D1 and A10. Since C1 had both the highest affinity of the three activators and also the greatest increase in PKCε activity, followed by D1 and A10, results of these experiments measured by different methods support each others. Of the two inhibitors (E6 and G8), E6 was a better binder of PKCε (Fig. 2, Table 1). As was the case with the activators, the obtained affinity constants support the results from kinase activity assays, where E6 is a more potent inhibitor.

Analyses of PKCε activation or inhibition by VHHs (Table 2) showed that the three PKCε activator VHHs have different mechanisms of activation. This suggests that they either increase the affinity to the substrate or the rate at which it is converted to the product. Based on the Michaelis-Menten constants obtained for the two PKCε inhibitory VHHs (E6 and G8), E6 is a more potent inhibitor.

In order to study whether the VHHs have an effect on the translocation of PKCε to the membranes, one of the activating (A10) and inhibiting VHHs (G8) were studied in HeLa cells. *In vitro* cell studies indicated that A10 increases the rate of PKCε translocation, whereas G8 slows it down [1].

Conclusions

Here the role of protein orientation in SPR measurements is emphasized by the finding that oriented immobilization of the target protein increases its immunobinding efficacy. The obtained affinity constants could be used for internal comparison to determine, which PKCε binders display the strongest interaction to PKCε. The results described here provide important additional information about the VHH activators and inhibitors of PKCε.

Here it is shown that kinetic studies *in vitro* by SPR can be used to predict the the activating/inhibiting function of anti-PKCε, and possibly other VHH antibodies.

VHH	k_a 1 (1/(M*s))	k_d 1 (1/s)	K_D 1 (M)	k_a 2 (1/(M*s))	k_d 2 (1/s)	K_D 2 (M)
A10	2.95×10^{1} ($\pm 4.31 \times 10^{2}$)	7.50×10^{-4} ($\pm 7.62 \times 10^{-5}$)	2.54×10^{-5} ($\pm 1.76 \times 10^{-6}$)	1.01×10^{3} ($\pm 1.98 \times 10^{3}$)	1.05×10^{-1} ($\pm 3.27 \times 10^{-6}$)	1.04E $\times 10^{-4}$ ($\pm 7.21 \times 10^{-5}$)
C1	3.09×10^{2} ($\pm 1.60 \times 10^{2}$)	1.04×10^{-3} ($\pm 3.56 \times 10^{-5}$)	3.38×10^{-6} ($\pm 2.56 \times 10^{-6}$)	1.12×10^{4} ($\pm 4.11 \times 10^{3}$)	8.21×10^{-2} ($\pm 4.10 \times 10^{-6}$)	7.30×10^{-6} ($\pm 3.08 \times 10^{-6}$)
D1	2.41×10^{1} ($\pm 2.89 \times 10^{-1}$)	1.07×10^{-3} ($\pm 2.00 \times 10^{-4}$)	4.42×10^{-5} ($\pm 8.80 \times 10^{-6}$)	5.51×10^{3} (± 1.27)	4.35×10^{-2} ($\pm 9.02 \times 10^{-5}$)	7.91×10^{-6} ($\pm 1.82 \times 10^{-8}$)
E6	5.22×10^{2} ($\pm 2.30 \times 10^{2}$)	3.06×10^{-4} ($\pm 6.60 \times 10^{-5}$)	5.87×10^{-7} ($\pm 4.78 \times 10^{-7}$)	1.53×10^{3} ($\pm 4.59 \times 10^{3}$)	1.49×10^{-2} ($\pm 5.10 \times 10^{-6}$)	9.71×10^{-6} ($\pm 3.64 \times 10^{-6}$)
G8	1.11×10^{1} ($\pm 7.11 \times 10^{2}$)	1.13×10^{-3} ($\pm 4.01 \times 10^{-5}$)	1.02×10^{-4} ($\pm 1.58 \times 10^{-6}$)	n/a	n/a	n/a

Table 1. Association and dissociation constants of VHHs with PKCε from SPR measurements. [3]

	PKCε with activators PS and DOG (n≥3)		PKCε without activators PS and DOG (n≥3)		Catalytic domain (n≥2)	
	K_m (µM)	V_{max}	K_m (µM)	V_{max}	K_m (µM)	V_{max}
Control	424	141	449	139	130	120
A10	348	253	352	263	no effect	no effect
C1	81	191	105	139	no effect	no effect
D1	126	158	137	208	no effect	no effect
E6	17	29	5.1	24	n/a	6.7
G8	260	113	121	71	158	43

Table 2. K_m and V_{max} values for VHHs activating and inhibiting PKCε. [4]

[1] Summanen et al., PloS ONE 2012, 7(4): e35630 ;
doi/10.1371/journal.pone.0035630
[2] Paalanen et al., Eur. J. Pharm. Sci. 2011, 42(4): 332-339.
[3] doi/10.1371/journal.pone.0035630.t001
[4] doi/10.1371/journal.pone.0035630.t002

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