Friday, March 20, Session V

L18

ANALYSIS AND PREDICTION OF PROTEIN SOLUBILITY

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The low solubility and poor expression of recombinant proteins are often a bottleneck in different biotechnological and pharmaceutical applications as well as in fundamental research, where high concentrations of purified protein are often essential. Poorly soluble proteins tend to aggregate. The presence of protein aggregates is associated with various neurodegenerative diseases. Therefore, understanding of the basis of protein solubility and expressibility and development of methods for their improvement are of great interest in many areas of research and development, including structural biology and biochemistry, industrial biotechnology and medicine.

An increase in protein's soluble expression can often be achieved using conventional methods, e.g., optimization of the host organism, modification of growth media, and expression at low temperature. An alternative approach is to modify the protein's amino acid sequence. Efforts have been made to apply artificial intelligence (machine learning) for predicting solubilizing mutations based only on the protein's amino acid sequence. However, currently available prediction tools are not very accurate and reliable [1]. The quality of machine learning predictions strongly depends on the size and quality of the experimentally acquired training data sets [2]. In this work, we introduce methods for high-throughput analysis of protein solubility, which are subsequently applied for the assessment of the effects of mutations on protein solubility. We adopted two general approaches, the split-GFP [3] and split-NanoLuc [4] technologies, and tested them in different experimental formats. The split-GFP system was used in combination with colony filtration using an immobilized bead assay [5], while the split-NanoLuc complementation approach was tested in a microtiter plate-based assay. Both methods are evaluated using a set of model proteins of varying soluble expression to assess their sensitivity, reliability and screening efficiency. The methods can provide the capacity to screen libraries of approximately 10^4-10^5 protein variants per screening campaign. The data collected will serve as a training set for the development of novel tools for the prediction of protein solubility.

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This project is supported by the Masaryk University grant MUNI/C/1647/2019.

L19

HOW PHOSPHORYLATION IMPACTS 14-3-3 DIMERIZATION

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The 14-3-3 proteins represent a large group of dimeric proteins. Specifically, the 14-3-3 family consists of 7 isoforms, that can form many homo- and heterodimeric states, not even accounting for the possibility of changing the oligomerization properties by posttranslational modifications such as phosphorylation. The functions of 14-3-3 are very often dependent on the dimeric state. Therefore, the parameters of oligomerization are very interesting in order to correctly understand the regulation of 14-3-3 itself.

In our study, we focused on the zeta isoform (most abundant isoform in human brain, also forming most stable dimers) and its phosphorylated form. Using standard biophysical methods we have only seen that the Kd is lower than 1 M. Therefore, we designed very sensitive fluorescence based methods to allow for study of such tightly bound dimers. Using these methods, we determined the dissociation constant, as well as kinetic parameters of the oligomerization process. Moreover, we studied the dependencies of the process on several buffer conditions, including temperature and ionic strength.

In order to determine the effect of phosphorylation on Ser58, which the literature is not sure about, we used our fluorescence assays. The serine S58 is located at the dimeric interface and therefore its phosphorylation affects the dimerization process. We succeeded in determination of both kinetic and equilibrium parameters of the interaction between non-phosphorylated and phosphorylated 14-3-3 protein.

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This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic within programme INTER-ACTION (project No. LTAUSA18168) and by the research grant from the Czech Science Foundation, grant no. GA15-34684L and GF20-05789L. Research infrastructure was supported by CEITEC 2020 (LQ1601) and the CIISB research infrastructure project supported by MEYS CR (LM2018127).

L20

PRELIMINARY CHARACTERIZATION OF HUMAN CD160 AND THE INFLUENCE OF VIRAL N-LINKED GLYCOSYLATION RELATED TO THIS IMMUNE INHIBITORY PATHWAY

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The CD160 (BY55) receptor was recently identified as a candidate target for immmunotherapy. Its expression is mainly, but not uniquely, restricted to cytotoxic cells, such as NKT cells and NK cells, and it is also present in T cells [1]. The relationship of human CD160 to HVEM (TNF receptor family member 14), and its viral ortholog UL144 (encoded by human cytomegalovirus, HCMV) are nowadays being extensively studied because of their specific properties in immune modulation. However, molecular characterization and binding properties of these molecules are not clear yet. Our biophysical and molecular characterization results provide insight into the basis for understanding of mechanisms by which CD160 modulates immune effector pathways that might be influenced by HCMV glycosylation.

Materials and methods

Construction of recombinant baculoviruses (bacmids) with target gene of CD160, was engineered by PCR and mature ectodomain of protein was cloned downstream of the gp67 signal sequence into the baculovirus transfer vector pAcGP67A containing an N-terminal Fc-tag. The recombinant bacmid with target gene was transfected into *Sf*9 cells to produce CD160 Fc-fused protein (about 52-60 kDa, Figure 1) via ProGreen™ Baculovirus DNA carrying a GFP reporter gene (AB Vector). After several rounds of viral amplification in *Sf9* cells, the recombinant protein was purify after 3 days from the cultivation medium of cultured *Sf9* cells infected with baculovirus according to previously reported conditions [2]. The purity, molecular mass, and

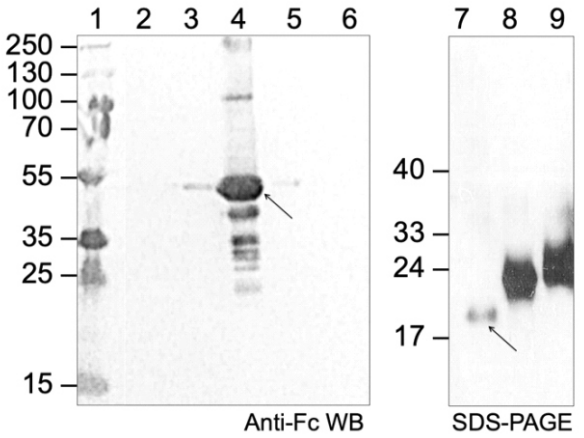

Figure 1. Western blot analysis of CD160 Fc-tagged protein using the Anti-Human IgG (Fc specific)-Peroxidase antibodies and SDS-PAGE analysis of HCMV UL144-mutants. Lane 1: PageRuler *Plus* Prestained *Protein Ladder* (Thermo Scientific); line 2: flow-through fraction, nonbinding proteins; lines 3-6: elution fractions; lines 7-9: mutants of HCMV UL144 with different glycosylation. The arrows indicate the CD160 Fc-tagged protein (52-60 kDa) and UL144-mutant (dg) used for binding assay.

the conformational changes of the protein were determined by various liquid chromatography (LC: FPLC), gel electrophoresis (SDS-PAGE) and western blot (WB) analysis (Figure 1). Kinetic binding studies of synthetized protein ectodomains were performed by surface plasmon resonance (SPR) equilibrium binding assays on BIACORE instrumentation and relative affinities were calculated. After

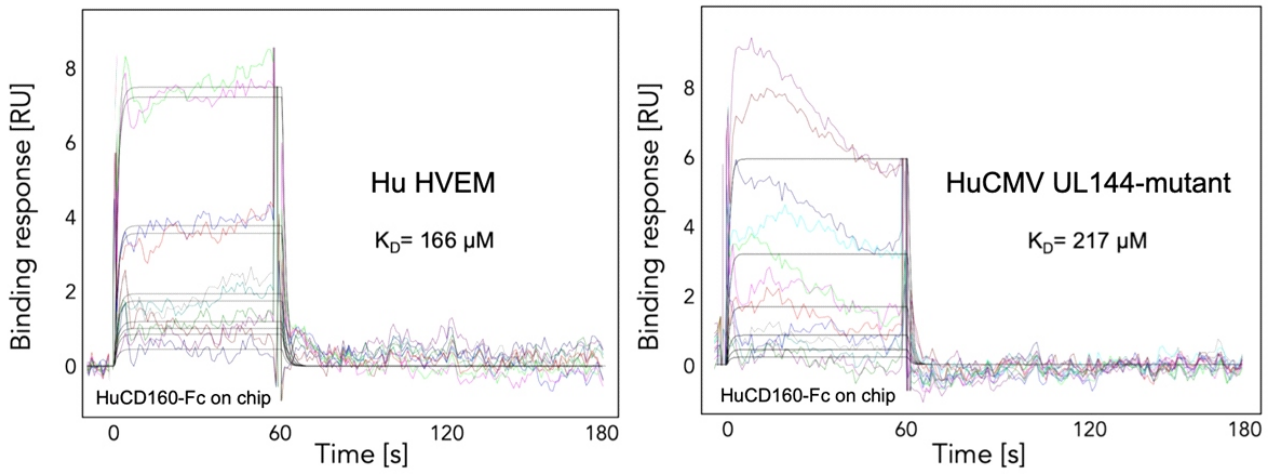

Figure 2. SPR analysis of HVEM-CD160 and dgUL144mutant-CD160 molecular complexes. Plots illustrating the experimental curve-fitting methodology for a simple binding model (1:1 Langmuir).

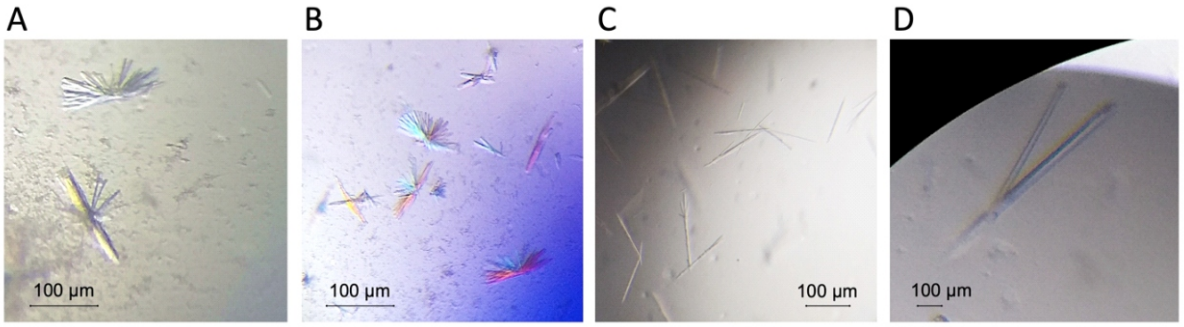

Figure 3. Preliminary crystallization of CD160-HVEM (A, B) complex and CD160 alone (C, D). Initial crystals were obtained under following conditions: 0.2 M ammonium chloride, 0.1 M HEPES pH 7.0, 20% PEG 6000 (Fig. 3 A, B); 0.2M sodium malonate dibasic monohydrate, 20 % PEG 3350 (Fig. 3 C); 0.2M sodium malonate dibasic monohydrate, 0.1 M Bis-Tris propane pH 6.5, 20 % PEG 3350 (Fig. 3 D).

a proper selection and establishment of the expression system followed by biochemical characterization at the protein level, we have started the initial screening for crystallization conditions in order to obtain suitable crystal for X-ray diffraction and structural studies.

Results and Discussion

CD160 was identified as a co-inhibitory molecule that binds HVEM with a similar affinity to human checkpoint receptor BTLA [3]. Our binding studies of expressed ectodomains revealed slower dissociation rate for HVEM-CD160 than for HVEM-BTLA molecular complex. Our recent data (unpublished) suggest that HCMV UL144, viral mimic of the HVEM, does not bind CD160, we hypothesize this is due to altered N-linked glycosylation. Mutant of UL144 (dgUL144) lacking these glycosylation sites (N61, N70, N73, N78, N91, N99, N116) was generated and we have determined whether its binding to CD160 is impacted. We have measured the binding kinetics (by using Biacore SPR assay) for the dgUL144-CD160 (K_D 217 M) and HVEM-CD160 (K_D 166 M) molecular complexes (Figure 2). Additionally, we have performed the crystallization condition screening for CD160 alone (thrombin digested Fc-portion) and HVEM-CD160 complex. The preliminary crystallization conditions were found (Figure 3) and will be further optimized and tested for X-ray diffraction that will provide critical insight into understanding of how CD160 function in regards to human HVEM and viral ortholog UL144 to regulate immune responses.

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Acknowledgements

The work was supported by the contribution of the Slovak Research and Development Agency under the project APVV-14-0839 and the contribution of the Scientific Grant Agency of the Slovak Republic under the grant 02/0020/18 and 02/0130/18. IN is Marie Curie Fellow financed by Programme SASPRO co-funded by European Union and the Slovak Academy of Sciences under the contract 0003/01/02. The part of the research team is supported by Interreg V-A SK-AT cooperation programme by project CAPSID under the contract No. NFP305010V235 co-financed by European Regional Development Fund.