CHARLES UNIVERSITY IN PRAGUE

THE FACULTY OF NATURAL SCIENCE
Department of Physical and Macromolecular Chemistry

The summary of the doctoral thesis

Study of interactions of forkhead transcription factor FOXO4 with DNA and the 14-3-3 protein

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1 Abstract

This doctoral thesis deals with the interaction of human forkhead transcription factor FOXO4 with DNA and regulating 14-3-3 protein respectively. The main aim of this work was detailed characterization of interaction between DNA binding domain of protein FOXO4 with two canonical DNA sequences and further clarifying the role of the 14-3-3 protein in the regulation of activity of protein FOXO4.

FOXO transcription factors are potent activators of the transcription of genes, which affect a variety of cellular processes. FOXO4 protein belongs to the family of forkhead transcription factor, which is a group of several tens of proteins, whose common feature is a highly conserved DNA-binding domain. Summary of the DNA binding specificity of these proteins, namely what precisely determines the small differences in the binding properties of individual forkhead proteins, despite the large amount of available structural data remains still unclear. Therefore, detailed characterization of interactions between DNA binding domain of the protein FOXO4 and DNA using surface plasmon resonance (SPR) and time-resolved fluorescence spectroscopy was performed. The results of this study allowed to clarify the kinetic model of FOXO4 binding to DNA as well as characterize both the conformational change of FOXO4 upon its binding to the target DNA and the importance of particular amino acid residues on the stability of the complex between FOXO4 and DNA.

The transcriptional function of FOXO4 is regulated by phosphorylation and binding of the 14-3-3 protein, which is by its nature regulator of many cellular processes. The mechanism of this regulation is still unclear. The 14-3-3 protein affects FOXO transcription factors in two ways. The complex formation inhibits FOXO4 binding to the target DNA and masks nuclear localization sequence (NLS), which consequently blocks the transport of FOXO proteins into the nucleus. Methods of fluorescence spectroscopy were used to study the interaction of these two binding partners. Time-resolved fluorescence of intrinsic as well as extrinsic fluorophores was used to show that the binding of the 14-3-3 protein affects multiple regions of the DNA binding interface of FOXO4. The results suggest that observed changes are induced by direct protein-protein interactions of these two binding partners. In addition, to better describe the interaction between these two proteins, a realistic structural model of the FOXO4:14-3-3 complex was constructed using six intermolecular distances obtained from Förster resonance energy transfer measurements. This model shows clearly that FOXO4 is located deep inside the central channel of the dimer of the 14-3-3 protein and its DNA binding interface is blocked by this interaction.

2 Introduction

The research, which this work was based on deals with the mechanism of interaction of the tirades of eukaryotic intracellular objects. These are proteins FOXO4, 14-3-3 protein and chromosomal DNA. These three objects together creates a system of mutually influencing elements. The main theme of this research is detailed description of the molecular mechanisms of interaction between these objects. Each of these objects is representative of a group of intracellular elements with a certain very important function.

Protein FOXO4 is representative of group of proteins also referred to as transcription factors. These proteins stands at the end of the signaling pathways and interacts directly with the chromosomal

DNA in the cell nucleus and thus activate transcription of genes involved in cell cycle regulation, cellular response to stress and the regulation of controlled cell death - apoptosis.
14-3-3 is a protein which contributes to the regulation of a variety of signaling pathways, thereby affecting a variety of cellular processes.

Nuclear chromosomal DNA, as one do not need to mention is the hereditary information bearer and thus most important of all

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2.1 FOX transcription factors

Transcription factors commonly known as "forkhead box" (FOX) are a group of proteins that are structurally and functionally defined among a broad range of different eukaryotic transcription factors. Their name is derived from the name of the gene described in popular model organisms, fruit flies (Drosophila melanogaster). This gene is very important for the development of the front and rear portion of the Drosophila embryo intestine, for the correct formation of the shape of the intestine respectively (Weigel D et al., 1989). All FOX proteins show a high degree of amino acid sequence identity in their "forkhead" DNA-binding domain (DBD) and creates a closed protein family in the subgroup of proteins with "winged" helices, which occur in prokaryotic as well as in eukaryotic cells (Clark KL, et al., 1993; Gajiwala KS, et al., 2000). In spite of the name origin, these proteins were first identified in humans. They were found in rearranged chromosomes of certain types of tumors (Galili N et al., 1993; Davis RJ, et al., 1994; Parry P et al., 1994; Borkhardt A et al., 1997; Hillion J et al., 1997; Anderson MJ, et al., 1998).

The impulse for further study of these transcription factors was the fact that they are very potent activators of transcription and thus are involved in a number of fundamental processes concerning cell cycle regulation. The processes that are influenced by "forkhead" transcription factors includes: energy metabolism, DNA damage repair, stress responses, cell division, differentiation, apoptosis, cell cycle control, and others.

The family of FOX proteins comprises of more than 100 known species, which are further divided into 17 subclasses (A to Q subclasses). All of these proteins contain a highly conserved DNA binding domain (DBD), also known as the so-called. "Forkhead box", hence the acronym FOX "or as the winged helix domain (winged-helix domain). This domain shows high degree of sequence conservation as well as it is very conserved towards its binding motifs and thus gives a high degree of interaction specificity which taken together creates fine instrument for regulation of genes that are linked to FOX proteins. The DBD of FOX protein contains approximately 110 amino acid long sequence composed of several alpha-helices, several beta sheets and two flexible actuators called wings (Weigel D et al., 1990; Kaestner KH, et al., 2000; Mazet F et al., 2003).

2.2 14-3-3 proteins

14-3-3 proteins form another highly conserved family of proteins whose importance in cellular biology has increased with the knowledge that these proteins participates in a wide range of fundamental cellular processes such as metabolism, protein transport, signal transduction, apoptosis and cell cycle regulation. Members of this protein family were first identified by Moore and Perez in 1967 and earned their common name from the designations used in the systematic classification of mammalian proteins of brain tissue (Moore BW et al., 1967). Since many of the 14-3-3 protein-protein interactions are phosphorylation dependent the 14-3-3 proteins are in close connection with the signaling pathways regulated by phosphorylation, which are essential for normal growth and

development of cells and whose dysfunctions or improper regulations might cause many serious diseases including uncontrolled tumor growth, ie cancer.

14-3-3 proteins are polypeptides of size range from 28 to 33 kDa, abundant in all eukaryotic organisms (Aitken A et al, 2006). When binding to its binding partner, they look for phosphoserin and phosphothreonin binding motifs. This kind of regulation system, the system based on the phosphorylation of a peptide as a ligand and the presence of a binding partner as an activator or repressor, is widely used in the cell, thereby giving large possibility to 14-3-3 proteins to participate as regulator of many cellular processes.

2.3 14-3-3ζ and FOXO4

14-3-3ζ regulatory protein is one of the most widespread types of mammalian isoforms of 14-3-3 proteins. Transcription factor FOXO4 is one of its most important binding partners. Together, they contribute for example in cell cycle regulation, together particularly they hold the cell in the G1 phase of the cell cycle.

The NMR structure of FOXO4 DNA binding domain published in 2001 (Weigelt J et al., 2001) shows that FOXO4 contains in its structure three phosphorylation motifs for protein kinase B (PKB) in the area of amino acid residues threonine 28, serine 193 and serine 258 (Tran H et al., 2003; Woods YL et al., 2002; Obsil T et al., 2003). Previous studies have shown that phosphorylation of FOXO4 at these sites leads to inhibition of the transcriptional activity of FOXO4 protein together with its export from the nucleus to the cytoplasm. Though simple phosphorylation of FOXO4 in itself is not enough for inhibition of transcriptional activity. It has previously been shown that for rapid export of the other transcription factor, FOXO3a, from the nucleus to the cytoplasm is necessary not only phosphorylation and its interaction with 14-3-3 protein, but also the presence or rather accessibility of the nuclear export sequence (NES) in its structure (Brownawell AM et al., 2001; Brunet A et al., 2002). A similar conclusion applies to the transcription factor FOXO4. It also contains the nuclear export sequence (NES) and furthermore a nuclear localization sequence (NLS) in its structure. To be successfully localized in the nucleus FOXO4 the presence of amino acid residues 180 to 221 was determined to be necessary. FOXO4 contains non-classical NLS, consisting of two parts which surrounds the PKB phosphorylation motif at the C-terminal DNA binding domain (serine 193). As was mentioned previously, phosphorylation of serine residue 193 which is located between two parts of the non-classical NLS of FOXO transcription factors, leads to a partial disruption of its function. Further inhibition is caused when binding of 14-3-3 protein to FOXO transcription factor due to steric hindrance of the NLS. According to current research, it appears that the 14-3-3 protein binding affects both parts of the NLS (Obsilova V et al. 2005). Nuclear localization sequence (NES) of FOXO4 protein is located in the section between 300. to 308. amino acid residuum. This area is very rich in lysine residues, which corresponds with sequences recognized by nuclear export system (Brownawell AM et al., 2001). According to these findings, we can conclude that 14-3-3 protein binding to FOXO transcription factors is not necessary just for their rapid export from the nucleus to the cytoplasm, but also for its very inhibition of its DNA binding properties. The exact molecular mechanism of inhibition of such DNA binding properties is still unclear (Obsil T et al., 2003).

3 Research aims

1) To characterize interaction between protein FOXO4 and 14-3-3 protein using fluorescence spectroscopy methods. To determine whether conformational change of FOXO4 happen upon complex formation and if that is so than in what areas.

2) To design a structural model of the FOXO4:14-3-3 complex based on the distances obtained by measuring FRET.

3) Perform a detailed analysis of the interaction of FOXO4 DNA-binding domain and DNA using directed mutagenesis method and surface plasmon resonance method.

4) To characterize structural changes of FOXO4 DNA-binding domain accompanying its binding to DNA using methods of time-resolved tryptophan fluorescence.

4 Methods

4.1 General methods

Methods referred here to as general were used for the preparation and validation of protein constructs for experimental measurements.

4.1.1 Preparation of the cDNA by restriction digestion

Preparation of the cDNA sequences of the genes encoding selected parts of the studied proteins was performed by PCR amplification of selected parts of genes associated with the introductions of specific cleavage sites into the sequence of the insert by PCR method. Further was the insert and the target plasmid adjusted by digestion at the unique cleavage sites. Subsequently, both components were ligated and verified by restriction digestion and sequencing.

4.1.2 Site directed mutagenesis using PCR

Site specific mutations in the DNA sequences of the studied proteins were introduced by the PCR method using primers where in otherwise complementary sequence has been based modified to get desired amino acid mutation.

4.1.3 Expression of proteins in a prokaryotic expression system

Protein expression was performed in *E.coli* expression strain BL21 (DE3). Expression was induced using IPTG at culture OD 0.6 to 0.8. Expression proceed for 15 - 20 h at 20 °C and a vortex speed of 200 rpm.

4.1.4 Protein purification using various types of chromatography

Protein constructs were purified using the affinity tags (His-tag, or GST), and further on basis of different physical-chemical properties, such as pI (cation and anion exchange) or size (gel permeation chromatography). Various types of chromatography were carried out gravitationally or on the instrument ÄKTA HPLC (Amersham Biosciences).

4.1.5 Mass spectrometry MALDI-TOF

Using this method we verified protein phosphorylation and labeling by fluorescent probe 1,5-IAEDANS. Measurement and analysis of mass spectrometry of all modifications was performed by

Ing. Miroslav Šulc, PhD. at the Institute of Microbiology AS CR using mass spectrometer BIFLEX (Bruker-Franzen).

4.2 Experimental methods

Methods referred here to as experimental were used for validation of protein constructs and for obtaining experimental data.

4.2.1 The surface plasmon resonance (SPR)

SPR method was used to examine the interaction of alanine mutants of FOXO4-DBD with dsDNA of motifs IRE and DBE in real time. ProteOn Instrument XPR36 Protein Interaction Array System (Bio-Rad, Hercules) measures the change of refractive index at thin gold layer on surface of so called chip. This change in refractive index is proportional to the instantaneous concentration of the substances on the chip surface. Thanks to this it is possible to collect data in real time and to obtain data needed to determine equilibrium dissociation constants (K_D), or the kinetic dissociation (k_D) and association constant (k_A).

4.2.2 Steady-state fluorescence measurements

These measurements were used to verify the binding properties of different types of complexes, which were the subject of experimentation. For this kind of measurement one of the complex elements has always been labeled with fluorescent probe. The concentration of this element in the solution was constant, while the second element of the solution was titrated. After each titration and after excitation of fluorescent probe by suitable wavelength fluorescence intensity was measured in two mutually perpendicular planes on fluorimeter Perkin Elmer LS50B. From the changes ratio of intensity of polarized radiation the fluorescence anisotropy at different points was determined and its change was evaluated.

4.2.3 Time-resolved fluorescence measurement

The basis of this method, which was used to examine changes in the area of fluorescence probe is repeated pulsed excitation of fluorescent probes, where the length of the excitation pulse is usually shorter than the fluorescence decay time, which allows to analyze the time dependence of the measured parameters. With this approach it is possible to monitor fluorescence decrease of the intensity over time that is relative and does not depend on the actual value of the fluorescence intensity, which is additionally dependent on the surroundings of the probe. Crucial observed variable is mean lifetime of the excited state of the molecule, "τ", ie. the time between excitation of the molecule (absorption of a photon) and light emission when returning to the ground state. The time decay is independent of the concentration of the sample and on the other hand dependent on the surrounding environment and thus can be used to determine the polarity of the environment, pH, temperature, ion concentration, or the presence of fluorescent quencher. Time-resolved fluorescence measurements were carried out in cooperation with Doc. RNDr. Petr Heřman, Ph.D. and Doc. RNDr. Jaroslav Večeř, Ph.D. from Faculty of mathematics and physics of Charles University in Prague. As measuring device was used instrument with a dye laser source (Spectra Physics model 375) and a photon counting detector with a photomultiplier (Hamamatsu, R3809U-50).

4.2.4 CD spectroscopy

CD spectroscopy method is based on the finding that a linearly polarized light may be decomposed into left-handed and right-handed circularly polarized light component. Both of these components pass through the different environment with slightly different speed, thereby cause rotation of plane polarized light. Both of these components are absorbed differently by optically active substances and the linearly polarized light is changed to elliptically polarized light. If we observe difference in extinction coefficients for left- and right-hand components we talk about circular dichroism (CD). CD spectra analysis was used to validate the native secondary structures of exemplary protein constructs.

5 Results and discussion

5.1 Preparation of protein constructs

Using the above described methods we prepared and validated protein constructs specifically designed for the planned experiments. The amount of purified protein constructs was usually between 5 to 10 mg of pure protein in concentration of 2-4 mg/ml. To verify unchanged native properties of proteins constructs (structural integrity and binding properties) binding essays and CD spectroscopy was performed for an exemplary protein constructs. According to results, gained by these methods, native properties of exemplary constructs were evaluated as unchanged, as shown on Figures 1 and 2.

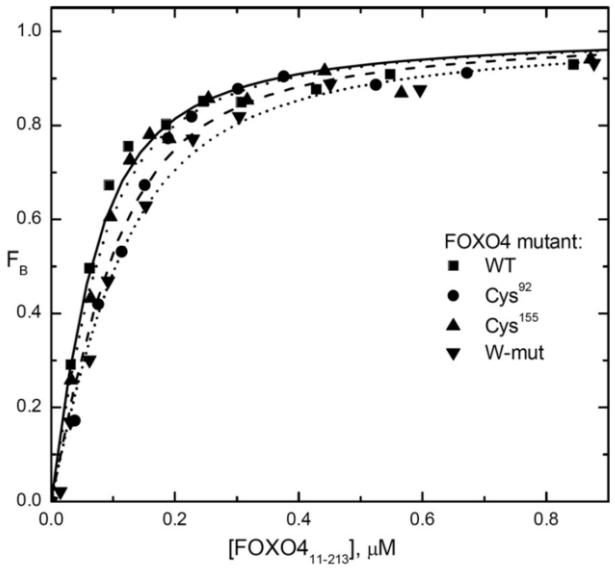

Fig. 1 Example of binding isotherms from the verification of the FOXO4$_{11\text{-}213}$ DNA binding properties. *When plotting parameter F_B (fraction of bound DNA) against concentration of titrated FOXO4$_{11\text{-}213}$ binding isotherm of individual constructs FOXO4$_{11\text{-}213}$ almost overlap with the binding isotherm of FOXO4$_{11\text{-}213}$ WT, indicating unchanged binding properties of compared protein constructs.*

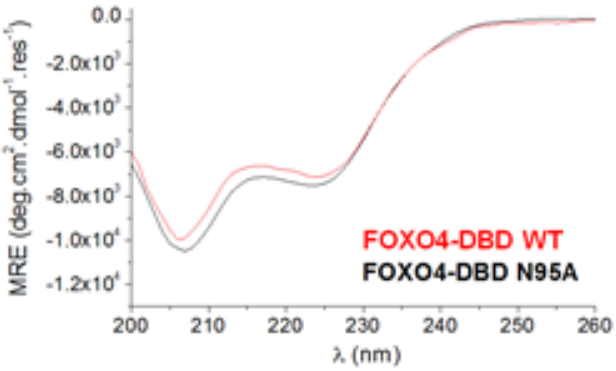

Fig. 2 Comparison of the CD spectra of the selected FOXO4-DBD protein construct and FOXO4-DBD WT. *Graphic representation of the results of the CD spectroscopy of selected FOXO4-DBD protein construct (mutant N95A) (black curve) shows compliance with the CD spectrum of FOXO4-DBD WT (red curve). It shows that the character of the curve does not change, thus the structure of the mutant FOXO4-DBD remains unchanged.*

5.2 Characterization of interaction molecular mechanisms of studied complexes

5.2.1 Characterization of the interaction between FOXO4-DBD and 14-3-3ζ

Time resolved fluorescence intensity decay measurements showed that the 14-3-3 protein binding affects the fluorescent properties of the AEDANS probe bound to four different locations in the structure of FOXO4-DBD, which represents an interesting area of DNA binding interface of protein FOXO4. From these observations, it is clear that changes in the fluorescent properties of the probe are caused by steric shielding of the fluorophore upon binding of 14-3-3ζ. This observation indicates the presence of physical contact between the labeled 14-3-3ζ and FOXO4-DBD. These observations are also consistent with the results of measurements of time-resolved fluorescence anisotropy (Fig. 3), showing that movement of certain segments of FOXO4-DBD is prevented when binding to the 14-3-3 protein.

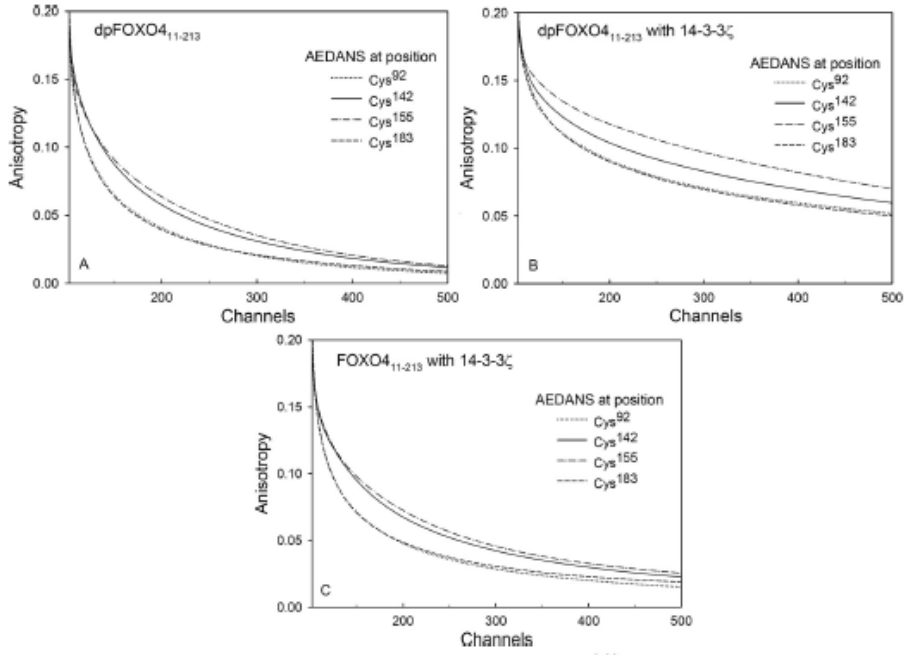

Fig. 3 Comparison of mobility of different FOXO4-DBD segments labeled by AEDANS probes. *Comparison of the fluorescence anisotropy decay records shows the mobility of individual fluorescent labeled segments. Individual types of lines represent the best fit of fluorescence anisotropy decay records.*

The observation of time-resolved tryptophan fluorescence of FOXO4-DBD shows that there are no significant conformational changes in the area of Trp173 and Trp174 upon its binding to protein 14-3-3ζ, which might have been a possible cause of the altered DNA binding affinity.

Another aim of the research was to create a structural model of the complex FOXO4:14-3-3ζ so we would be able to map the interactions between FOXO4 and 14-3-3ζ more clearly. For this purpose we determined six intermolecular distances in the complex FOXO4-DBD:14-3-3ζ using FRET method. This model of pFOXO4:14-3-3ζ (Fig. 4) shows that FOXO4-DBD is embedded in the central canal of the dimeric protein 14-3-3ζ in the same manner as has been published in previous studies concerning 14-3-3ζ protein in complex with serotonin N-acetyltransferase (Obsil T et al., 2001). This position allows effective coverage of the entire DNA binding interface of FOXO4 protein which is in agreement with the results of our fluorescence data. Another aspect of the interaction, which is also in agreement with our fluorescence data and also with previously published results (Obsilova V et al., 2005) is observation of almost unchanged mobility of DNA-binding domain of FOXO4 in complex with 14-3-3ζ protein.

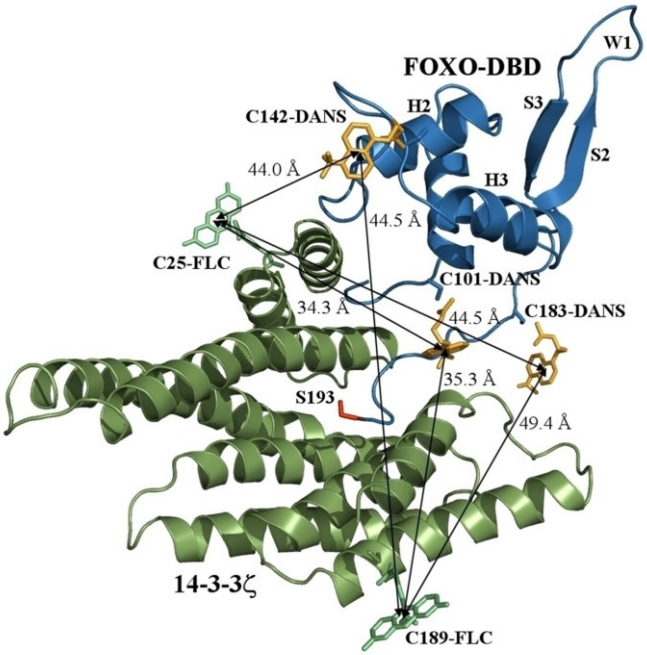

Fig. 4 The structural model of the complex FOXO4-DBD:14-3-3ζ. *The structural model of the FOXO4-DBD:14-3-3ζ complex offers better idea of the relative positions of the studied proteins, thus allowing a clearer interpretation of the results of other measurements.*

5.2.2 Characterization of FOXO4-DBD DNA binding mechanism

The kinetic parameters of interaction of FOXO4-DBD alanine mutants with dsDNA (dsDNA with motifs IRE or DBE) were determined from a concentration-dependent binding curves obtained by SPR method (Fig. 5). To obtain the association and dissociation rate constants, data were confronted first with simple Langmuir 1:1 model and also with a two-state model which takes into account also the conformational changes. A two-state model was evaluated as more relevant based on a better parameter of reduced χ^2. This means that such a two-state model describes the process of association and dissociation of the complex FOXO4-DBD:dsDNA more precise. The values determined for association (k_{a1}, k_{a2}) and dissociation (k_{d1}, k_{d2}) rate constants for the both binding DNA motifs DBE and IRE show that FOXO4-DBD WT binds both dsDNA with a K_D in the nanomolar range with binding affinity for DBE motif about four times higher than for motif IRE, which is entirely consistent with previously published data (Furuyama T et al., 2000; Brent MM, et al., 2008). Overall comparison of the K_D values indicates significant decrease in binding affinity (K_D increase) for several FOXO4-DBD mutants with mutations in different parts of the DBD. In case of the dsDNA motif IRE, the largest decline was recorded in binding affinity of mutants Y102F, N148 and ΔC, while binding to dsDNA motive DBE showed the greatest decline for mutants S101, Y102F, N148, H152A, K162 and ΔC. In agreement with previous studies, we observed significant differences in the N-terminal area of the protein FOXO4 and within the loop region between helices H2 and H3 and in the area of wings W1 and W2 (Tsai KL et al., 2007; Boura E et al., 2010; Weigelt J et al., 2001; Wang F et al., 2008).

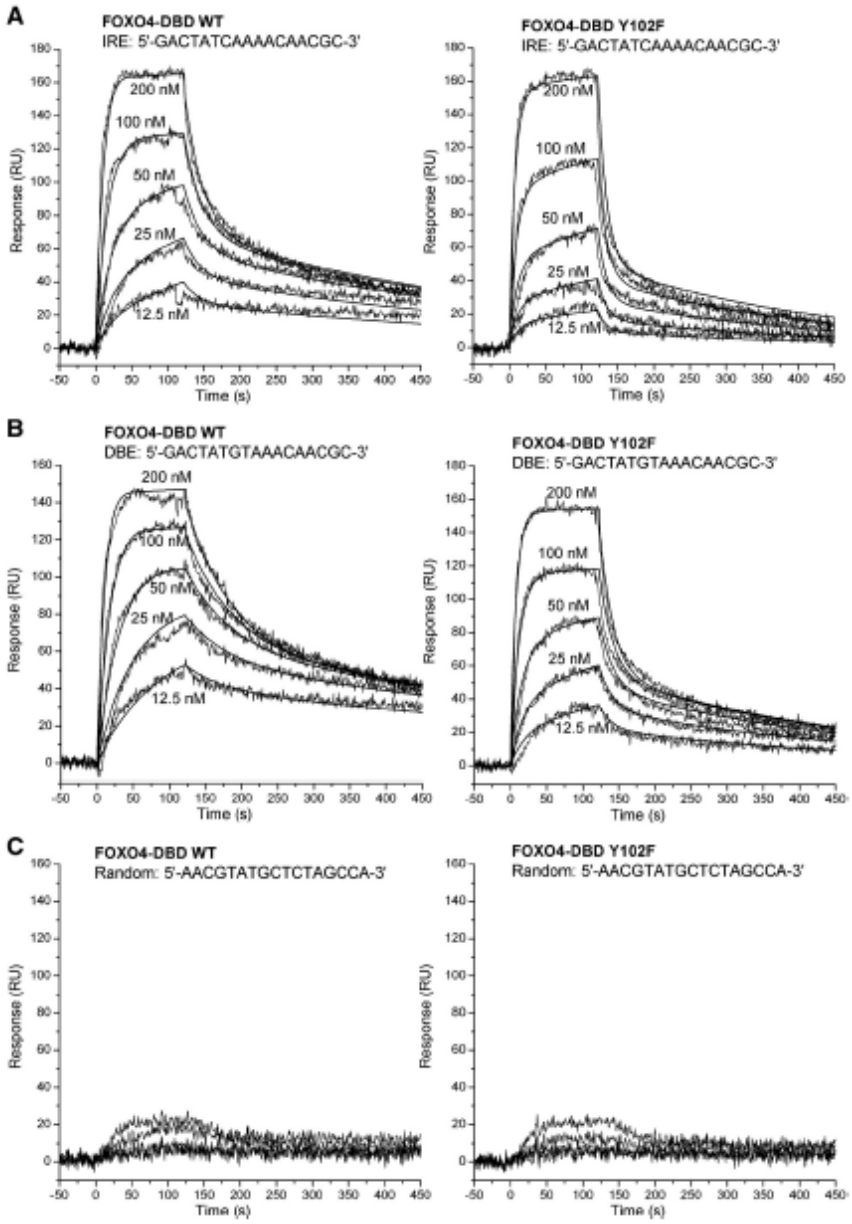

Fig. 4 SPR sensorgrams obtained by the measurement of kinetics of interaction of FOXO4-DBD and DNA. *SPR Sensograms represent comparison of data for different concentrations of the selected mutant compared to the WT form. Each separate sensorgram curve has three phases. Phase with positive slope representing the association of complex, "plateau" phase, and phase with a negative slope for the dissociation of the complex. From the character of these three phases one can see the nature of interaction and extrapolate values of the association and dissociation constants as well as the value of equilibrium dissociation constant. Part A of the picture represents comparison of interaction of FOXO4 WT and construct Y102F upon binding to DNA motif IRE. Part B represents comparison of interaction of FOXO4 WT and construct Y102F up on binding to DNA motif DBE. Part C of the picture represents comparison of interaction of FOXO4 WT and construct Y102F up on binding to random DNA motif.*

Conformational changes observed by SPR were confirmed by measuring the time-resolved fluorescence anisotropy decay of tryptophan residues of FOXO-DBD. A significant change in the value of the sum of the amplitudes corresponding to quick movement β1 + β2 and absence of rotation correlation time ϕ2 representing segmental motions of protein molecules when bound to DNA shows

a significant decrease in mobility of the studied segments and thus the changes in the structure of the molecule. These results therefore confirm the two-state binding model presuming conformational change of FOXO4-DBD upon binding to DNA.

6 Conclusion

The conclusion of this work could be summarized as follows:

1) Using fluorescent spectroscopy revealed that 14-3-3 protein interacts with several of the DNA binding segments of protein FOXO4. Specifically, there are close interactions in the areas of helices H2 and H3, the N-terminal segment and the wings W1 and W2. It was further found that the complex formation induce a structural change within the DNA binding domain.

2) Using FRET method was measured 6 intermolecular distances within the FOXO4:14-3-3 complex. These distances were used to design structural model of this complex. This model confirmed that 14-3-3 protein blocks a substantial portion of the DNA binding interface of protein FOXO4.

3) Using site directed mutagenesis, a series of alanine mutants of FOXO4-DBD was prepared and using surface plasmon resonance method detailed analysis of the interaction of FOXO4-DBD and DNA was performed. This analysis showed the importance of various non-covalent interactions for binding FOXO4-DBD to DNA, and also helped to establish a kinetic model of the interaction involving conformational change of FOXO4-DBD.

4) Based on data obtained by time-resolved tryptophan fluorescence measurement of FOXO4-DBD when bound to DNA, we were able to characterize the structural changes in the FOXO4-DBD that accompany this interaction.

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8 Curriculum vitae

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Education	2000 – 2006	**Charles University of Prague Faculty of Science** Master degree programme, subject Teaching & Didactics of Chemistry and Biology.
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Professional experience	2006 – 2008	**APRONEX s.r.o** **Applied scientist** Preparation of recombinant proteins in prokaryotic and eukaryotic expression systems.
	2003 – 2012	**INSTITUTE OF PHYSIOLOGY AS CR** **Graduate expert worker** Characterization of protein complexes.
	2013 - still	**SEQme s.r.o. Seq & qPCR Company** **Lab scientist** DNA and RNA analysis by 1st a 2nd generation sequencing methods.

Publications and lectures

Vacha P., Silhan J., Vecer J., Herman P., Sulc M., Obsilova V., Obsil T. Study of the interactions between 14-3-3 protein and DNA-binding domain of forkhead transcription factor FOXO4
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Training courses and internships

RNA-seq Bioinformatics Workshop 2014 (Course)
16. - 17.10 2014 / ecSeq Bioinformatics / Leipzig / Germany
(analysis of sequencing data from NGS sequencers)
2015 Workshop on genomics (Course)
11. - 23.1 2015 / Evomics / Český Krumlov / ČR
(analysis of sequencing data from NGS sequencers)
Roy J. Carver Biotechnology Center University of Illinois (internship)
2.- 3.4. 2015 / DNA service lab - Alvaro Hernandez Ph. D./Champaign-Urbana/Illinois
(preparation of sequencing libraries for MiSeq and HiSeq2500 sequencers)