Abstract

Life is a continuum of self-sustaining information – information in the form of a genetic code saved in DNA molecules. Maintaining the information intact and in the proper form for secure transfer to other generations is the overarching goal of every living entity. In order to meet these aims organisms developed sophisticated mechanisms for keeping their genome safe from DNA damage that arise as a result of a plentitude of exogenous and endogenous sources, like solar radiation or recombination processes during meiosis, respectively. The most deleterious damage of all are the double-stranded DNA breaks (DSB), that if left unattended, could lead to gross chromosomal aberrations and even cell death. The MRN/X complex is responsible for DSB recognition and repair and is a protein machinery that, either in the form of heterotetramer ($Mre11_2Rad50_2$) or heterohexamer ($Mre11_2Rad50_2Nbs1_2$/$Xrs2_2$ (Xrs2 – Nbs1 homologue in *S. cerevisiae*)), is utilized by all known living organisms. The Rad50 protein, which belongs to the structural maintenance of chromosomes (SMC) protein family, forms a structural core of the complex. It has a unique, bipolar architecture consisting of the globular ATP-ase domain responsible for DNA binding, and the zinc hook domain responsible for dimerization, that are separated by a coiled-coil segment of approximately 500 Å in length. Such topology enables bridging two broken DNA molecules separated in nuclear space even by a distance of 1200 Å. In order to begin the DSB repair broken strands need to be optimally allocated for one out of two main DSB repair routes – either a precise mechanism of homologous recombination that necessitates a template in the form of sister chromatid, or fast, yet more error-prone, pathway of non-homologous end joining. Ability to accommodate these two vastly different DNA repair approaches imposes a certain structural and functional flexibility on the MRN/X complex, that enables the optimal choice of repair pathway depending on the break itself, as well as cellular status. Here, the bipolar nature of the Rad50 protein unveils itself – now in the form of MRN/X complex's regulation of the structure-function relationship. Both the globular domain, as well as the zinc hook domain, are responsible for the complex functional status and allosteric changes that are generated at the one apex of the protein are transferred to the other one, and vice versa.

The zinc hook domain (Hk) is the prime example of an inteprotein zinc binding site. It consists of two evolutionary conserved CXXC motifs coming from two Rad50 protomer that together form a ZnS_4 coordination sphere of tetrahedral geometry. Formation of the $Zn(Hk)_2$ complex, as well as interaction of the globular domains at the other apex of Rad50, maintains the dimeric assembly of the MRN/X complex. Although stability of the zinc hook complex is extremely high (highest formation constant of a biomolecule complex with Zn(II) reported to date) a long segment of coiled-coil structure adjacent to the zinc hook domain, hinders the precise biophysical analysis of this domain. The first, and for several years the only, crystal structure of the zinc hook domain showed the central

fragment of the Rad50 protein from the hyperthermophilic organism of the archaea *P. furiosus*. The symmetrical, open structure of the dimer, however, did not explain all the complex arrangements observed in the studies of atomic force microscopy and electron microscopy. In addition, differences in the amino acid sequence closely adjacent to Zn (II) binding residues, as well as in further regions of the coiled-coil segment, between Rad50 proteins from different organisms suggest that the hook domain structures may present a significant level of heterogeneity in organisms across different domains of life. One particular factor that could be responsible for such diversification is the additional Cys residue located on the amino side of the first Cys residue of the binding motif (**C**C<u>C</u>XX<u>C</u>) in the Rad50 sequences from majority of eukaryotic organisms, which could potentially be involved in Zn (II) binding and influence the structure of the Zn(II) coordination sphere.

This dissertation describes the study of the zinc hook domains of Rad50 proteins from three organisms - *H. sapiens*, *S. cerevisiae* and *P. furiosus* – with the aim to provide new biophysical data shedding light on the following issues: i) what is the structure of the zinc hook domain in proteins of eukaryotes, ii) what is the role of the third Cys residue within the Zn (II) binding motif, iii) how the zinc hook domain can transmit allosteric signals to the globular part of the MRN/X complex (and in the other direction) and iv) whether the Zn ion (II) in the hook complex can be substituted by the toxic Cd (II) ion - which would explain the genotoxic potential of this ion.

The first part of this thesis describes the structural studies of the zinc hook domain of the human Rad50 protein. Cooperation with the group of prof. Yunje Cho, which resulted in solving the crystal structure of the domain, allowed to show a new arrangement of Rad50 protomers in a zinc hook dimer - a closed conformation, in a rod-shaped assembly. The closed dimeric form seems to be maintained by additional dimerization interfaces between the protomers - the hydrophobic and electrostatic interface. *In vitro* studies using hetero-FRET spectrofluorimetric analysis confirmed the *in vivo* analyzes performed on *S. cerevisiae* cells and together showed that newly discovered interfaces in the human hook domain are responsible for maintaining closed dimer conformation. Moreover, the research carried out with the use of appropriately designed mutational variants of the zinc hook domain proved that the new electrostatic interface and the coordination interface - the Zn (II) binding motif - are interdependent and cooperate in the functional regulation of the MRN/X complex.

The second part of the thesis describes the research on the Rad50 zinc hook domain from *S. cerevisiae*. Extensive biophysical analysis of the domain, carried out on domain fragments ranging from 5 to 196 amino acid residues in length, showed that the structure of the yeast zinc hook exhibits an open dimer conformation, in contrast to that of the human homolog, and is closer in resemblance to the classical structure of the *P. furiosus* protein. Additionally, significant structural changes in the domain were recorded under the influence of increasing concentration of Zn(II) ions. Detailed

analysis of this process using precise buffering of Zn(II) showed that the yeast zinc hook binds two Zn(II) with significantly different affinities, and the binding of each of them brings about global changes in the structure of the peptide chain. Thanks to the studies of a mutated variant of the hook domain in the form of an additional Cys residue substitution, which was replaced with a Gly residue (C686G), it was proved that the additional residue is responsible for the described structural dynamics of the hook domain. Studies showed that by providing an additional S^- donor C686 residue allows for the coordination of the second Zn(II) ion in the mononuclear $Zn(Hk)_2$ hook complex and the formation of the binuclear Zn_2S_6 center, thereby generating the $Zn_2(Hk)_2$ complex. The transformation of the mononuclear form to the binuclear form of the zinc hook domain involves the scissor-like movement of the Rad50 protomers' super-helical regions - from open to closed form - and suggests the possibility of zinc regulation of the structure and function of the entire MRX complex in yeast cells.

The third and last part of the thesis concerns the influence of the toxic Cd II) ion on the zinc hook domain of the Rad50 protein. Cd(II), besides imposing a number of highly toxic effects on cells exposed to it, is a carcinogen and an ion with genotoxic potential, the mechanism of which is still unknown. Due to the high affinity of ligands with sulfur donors for Cd(II), it efficiently displaces Zn(II) from zinc proteins engaging Cys residues at metal ion binding sites, resulting in generation of a toxic excess of free Zn(II) in the cell and disrupting homeostasis of the zinc proteome. Because Rad50 protein harbors tetracysteine Zn(II) binding site and is fundamental to DNA repair processes it is a potential target for the toxic Cd(II), and the Zn(II)/Cd (II) exchange in the zinc hook domain may be responsible for its genotoxic effects. The analysis of Zn(II)/Cd(II) exchange in the hook domain of the Rad50 from *P. furiosus* showed that the exchange of ions occurs quickly and efficiently, even in the presence of metallothioneins - Zn(II) storage proteins in cells with high affinity for Cd(II), and the complex retains its stoichiometry ($Zn(Hk)_2 + Cd(II) \rightleftharpoons Cd(Hk)_2 + Zn(II)$). Thermodynamic studies of the exchange process have shown that the main driving force of the process is a favorable change in the enthalpy of Cd—S bond formation, which is as much as 2.6 kcal/mol lower (thermodynamically more favorable) than the enthalpy of Zn—S bond formation. The resulting $Cd(Hk)_2$ complex is the most stable complex of the Cd(II) ion with a biological molecule described in the literature ($-\log K_d = 22.7$), and its structure is significantly different from that of the zinc complex, as documented by nuclear magnetic resonance structural analysis . The results obtained in the course of the research suggest that Zn(II)/Cd(II) exchange in the zinc hook domain may occur in cellular conditions, which may result in impaired Rad50 function and, as a result, weakening of DSB repair mechanisms, causing a genotoxic effect.