Project description

Genetic diseases might be cured by the correction of the mutated gene without otherwise modifying the genome. A promising strategy for this is to utilize the cell's own repair machinery in a homologous recombination (HR) process. In this approach, the correct DNA sequence is supplied and it replaces the deficient gene. A major obstacle has been the fact that the natural recombination events occur at a low frequency of ~10^{-6}. A double-strand break at or near the target sequence introduced by engineered specific nucleases, however, increases the frequency of HR by several orders of magnitude. [1]

Therefore, ***the goal of our research is to design and prepare a new artificial, DNA sequence-specific metalloendonuclease, being safe in a term that it should not exert any cytotoxicity arising from cleavage at off-target sites.*** In such chimeric nucleases a domain responsible for non-specific nuclease activity (containing a metal ion in its active centre) is linked to a DNA-binding protein with known or designed DNA sequence-specificity (Figure 1.)

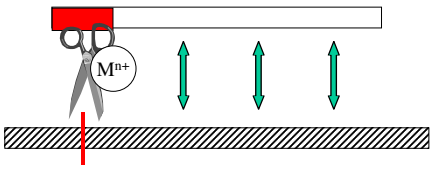

Figure 1. *The schematic representation of artificial metallonucleases*

State of the art

Since metal ions often take part in the catalysis of hydrolytic reactions in nature, bioinorganic chemistry, as an interdisciplinary research field, has also addressed efforts toward the development of artificial metallonucleases (AMNs). A large number of model coordination compounds have been tested against activated or natural macromolecular substrates, and were found to be efficient catalysts. [e.g. 2-7] In the Biocoordination Chemistry Research Group of the Department of Inorganic and Analytical Chemistry, University of Szeged, Hungary, it is also a hot topic. [e.g. 8-15] However, small molecular models are difficult to turn into a highly specific nuclease. Therefore, several other strategies have been applied to develop artificial nucleases (ANs) from natural proteins. Among these, the most well-known ones are the mutation of the wild-type enzymes, the directed evolution method and the construction of chimeric enzymes. [1]

The discovery of the restriciton endonucleases in 1970 [16] – awarded by Nobel prize in 1978 – taught us, among others, that many of these enzymes contain well defined separate domains for DNA recognition and for cleavage. Such peptide or protein fragments possess the advantage that they can easily be fused to the amino acid sequence of another protein by means of recombinant DNA technology, resulting in new chimeric enzymes – modular enzymes constructed from functional domains of different proteins.

Metal ion complexes of oligopeptides of ~ 40 amino acids may function either as catalytic or recognition modules in such an enzyme. E.g. the only promising chimeric ANs today are represented by zinc-finger nucleases (Figure 2.). Their recognition site consists of zinc-finger domain(s) with a conformation suitable to bind and specifically recognize unique nucleic acid sequences upon metal binding. Each zinc-finger motif is built up from two antiparallel β-strands and an α-helix connected by a loop structure. The consensus sequence is the following: (Tyr,Phe)-X-Cys-$X_{2,4}$-Cys-X_3-Phe-X_2-HSt-X_2-Leu-X_2-His-X_{3-5}-His, where the amino acid at the start of the α-helix is marked by HSt. The metal binding occurs by the conserved two cysteines and two histidines. A zinc-finger motif recognizes three bases in the DNA sequence by means of specific contacts through the amino acid side-chains at positions –1, 1, 2, 3, 5, and 6, (numbered relative to the start of the α-helix) [17]. By the variation of

these residues in an array of zinc-finger motifs, they may be designed to recognize any unique sequence even in the DNA of the size of human genome.

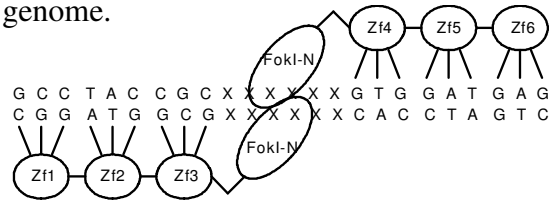

***Figure 2**. The dimerization of a zinc-finger nuclease upon binding to the DNA substrate*

The today's zinc-finger nucleases, since their invention, apply exclusively the non-specific catalytic domain of the FokI restriction endonuclease. [18] They function upon head-to-head dimerization on the substrate. By such ANs efficient targeting of different genes *in vivo* became possible. *Drosophila, Xenopus laevis, Caenorhabditis elegans, Arabidopsis* and mouse genes were targeted for mutagenesis. It has also been discovered, that a specific chromosomal cleavage by zinc-finger nucleases in plants and even in human genome [19-21] increases the frequency of the HR events up to 50%, thus new or missing genome sequences can be introduced into the cells' genetic material.

This concept might give a hope for children born with lethal genetic diseases, as it is e.g. the Duchenne Muscular Dystrophy [22-24], where in most cases the deletion of one or more exons from the DMD gene occurred. Introducing the appropriate DNA sequence as a template and the specific nuclease cleavage near the breakpoint of the gene (the sequence of the recombined gene at the mutation), the DNA-repair machinery of the cells may recover the functional gene for dystrophin expression. For such a therapeutic action it is necessary for the nuclease to be extremely specific, not to cause any side effect in the targeted organism. The zinc-finger nucleases, however, exert a minor cell toxicity, due to the cleavage at off-target sites. [25] This was supposed to be due to the possibility of the homodimerization instead of the heterodimer formation resulting in unwanted site recognition. In order to avoid this side reaction, the dimerization surface of the nuclease domain has been modified, showing perfect results in test systems, but not in cells. [26,27]

Methodology

Within the frame of the work the new genes of the nuclease and zinc-finger domains, as well as the target sequences will be designed, prepared and sequenced within different plasmid vectors providing different fusion tags for enhanced expression and easy purification. Such vectors are the pGEX-6P-1 (Amersham Biosciences - with an N-terminal gluthathione-S-transferase - GST - fusion), the pMAL-b vector constructed from the pMAL-c2x (New England Biolabs - with an N-terminal maltose binding - MB – protein fusion) and the pET-21a vector (Novagen - with a C-terminal hexahistidine, if appropriate) The genes of the hydrolytic units within the plasmid vectors will function as fusion tags to any nucleic acid-binding proteins - representing a useful delivery of the present project.

Optimization of expression from different vectors in suitable cell lines, under different conditions, and the purification of the AMN proteins by affinity chromatography, will constitute the next major part of the project.

Further, the association constants of the AMNs with metal ions and DNA (also when the zinc-finger array is already built in) and the structural influences will be determined by means of fluorescence, CD, NMR spectroscopy, MS and gel electrophoresis methods (e.g. gel shift) under optimized conditions.

The computer design and docking experiments will be combined with affinity selection of a zinc-finger domain specific to the selected target DNA sequence. The latter will be performed in the form of a phage display, where the proteins are expressed on the surface of a phage, and selected by e.g. a biotinylated target DNA sequence.

The obtained artificial enzymes will be tested for the catalytic activity under optimized conditions. The target and sequence specificity of the enzymes will be further assayed using DNA mixtures. The fine tuning of the activity will also be performed. In these experiments a zinc finger array with known specificity will be applied as a control. [27] The target AMNs shall perform a specific cleavage at the target sequence, i.e. close to the breakpoint in a Duchenne patient's DNA, and induce the HR process in a simple test system to be elaborated.

Literature

[1] A. Pingoud, G.H. Silva: Precision genome surgery. *Nature Biotechnol.* **25,** 743-744 (2007).

[2] B. Gyurcsik: Chimeric Metallonucleases-Design, Realization and Applications, *In: Inorganic Biochemistry: Research Progress*. Eds. J.G. Hughes and A.J. Robinson, 2008, Ch. 1, pp. 11-26, Nova Science Publishers, NY, ISBN: 978-1-60456-708-3

[3] R. Krämer, T. Gajda: Functional model complexes for dinuclear phosphoesterase enzymes, *In Perspectives on Bioinorganic Chemistry,* Ed. R.W. Hay, J.R. Dilworth, K.B. Nolan, Eds., 1999, 4, pp. 207-240, JAI Press Inc., Stanford.

[4] J.R. Morrow, O. Iranzo: Synthetic metallonucleases for RNA cleavage. *Curr. Opin. Chem. Biol.,* **8,** 192-200 (2004).

[5] J.A. Cowan: Chemical nucleases. *Curr. Opin. Chem. Biol.,* **5,** 634-642 (2001).

[6] E. Kimura: Dimetallic hydrolases and their models. *Curr. Opin. Chem. Biol.*, **4,** 207-213 (2000).

[7] N.H. Williams, B. Takasaki, M. Wall, J. Chin: Structure and nuclease activity of simple dinuclear metal complexes: quantitative dissection of the role of metal ions. *Acc. Chem. Res.,* **32,** 485-493 (1999).

[8] I. Török, T. Gajda, B. Gyurcsik, G.K. Tóth, A. Péter: Metal complexes of imidazole ligands containing histamine-like donor sets: equilibrium, solution structure and hydrolytic activity. *J. Chem. Soc., Dalton Trans*., 1205-1212 (1998).

[9] B. Gyurcsik**:** Mesterséges metalloenzimek tervezése, előállítása és vizsgálata, *Biokémia*, **29**, 37-42 (2005)

[10] N.I. Jakab, A. Jancsó, T. Gajda, B. Gyurcsik, A. Rockenbauer: Copper(II), nickel(II) and zinc(II) complexes of N-acetyl-His-Pro-His-His-NH_2: equilibria, solution structure and enzyme mimicking. *J. Inorg. Biochem.*, **102,** 1438-1448 (2008)

[11] N.I. Jakab, O. Lőrincz, A. Jancsó, T. Gajda, B. Gyurcsik: Approaching the minimal metal ion binding peptide for structural and functional metalloenzyme mimicking. *Dalton Trans.,* 6987-6995 (2008).

[12] A. Kolozsi, I. Vosekalna, T. Martinek, E. Larsen, B. Gyurcsik: Copper(II) and zinc(II)ion binding properties of a MAP type branched ligand with histidines as surface functionalities. *Dalton Trans.,* (2009), DOI: 10.1039/b903721g

[13] Q. Wang, E. Leino, A. Jancsó, I. Szilágyi, T. Gajda, E. Hietamäki, H. Lönnberg: Zn^{2+} Complexes of Di- and Tri-nucleating Azacrown Ligands as Base Moiety Selective Cleaving Agents of RNA 3',5'-Phosphodiester Bonds: Binding to Guanine Base. *ChemBioChem.,* **9,** 1739-1748, (2008).

[14] A. Jancsó, S. Mikkola, H. Lönnberg, K. Hegetschweiler, T. Gajda: Hydrolysis of a mRNA 5′-cap model substrate, 5′,5′-ApppA by di- and trinuclear zinc(II) complexes of a polyamino-polyol ligand. *J. Inorg. Biochem.,* **99,** 1283-1293 (2005).

[15] A. Jancsó, S. Mikkola, H. Lönnberg, K. Hegetschweiler, T. Gajda: Phosphodiester cleavage of ribonucleoside monophosphates and polyribonucleotides by homo and heterodinuclear metal complexes of a cyclohexane-based polyamino – polyol ligand. *Chem. Eur. J.,* **9,** 5404-5415 (2003).

[16] H.O. Smith; K.W. Wilcox: A Restriction Enzyme from Hemophilus influenzae. *J. Mol. Biol.,* **51,** 379-391 (1970).
[17] D. Jantz, B.T. Amann, G.J. Gatto, Jr., J.M. Berg: The design of functional dna-binding proteins based on zinc-finger domains. *Chem. Rev.,* **104,** 789-799 (2004).
[18] Y.-G. Kim, J. Cha, S. Chandrasegaran: Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. USA*, **93,** 1156-1160 (1996).
[19] E.A. Moehle, J.M. Rock, Y.-L. Lee, Y. Jouvenot, R.C. DeKelver, Ph.D. Gregory, F.D. Urnov, M.C. Holmes: Targeted gene addition into a specified location in the human genome using designed zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA,* **104,** 3055-3060 (2007).
[20] A. Lombardo, P. Genovese, C.M. Beausejour, S. Colleoni, Y.L. Lee, K.A. Kim, D. Ando, F.D. Urnov, C. Galli, P.D. Gregory, M.C. Holmes, L. Naldini: Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nature Biotechnol.*, **25,** 1298-1306 (2007).
[21] V.K. Shukla, Y. Doyon, J.C. Miller, R.C. DeKelver, E.A. Moehle, S.E. Worden, J.C. Mitchell, N.L. Arnold, S. Gopalan, X. Meng, V.M. Choi, J.M. Rock, Y.Y. Wu, G.E. Katibah, G. Zhifang, D. McCaskill, M.A. Simpson, B. Blakeslee, S.A. Greenwalt, H.J. Butler, S.J. Hinkley, L. Zhang, E.J. Rebar, P.D Gregory F.D. Urnov: Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature,* **459,** 437-441 (2009).
[22] G. Cossu, M. Sampaolesi: New therapies for muscular dystrophy: cautious optimism. *TRENDS Mol. Med.,* **10,** 516-520 (2004).
[23] J.S. Chamberlain: A move in the right direction. *Nature,* **444,** 552-553 (2006).
[24] K.J. Nowak, K.E. Davies: Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO Rep.,* **5,** 872-876 (2004).
[25] T.I. Cornu, S. Thibodeau-Beganny, E. Guhl, S. Alwin, M. Eichtinger, J.K. Joung, T. Cathomen: DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol. Ther.*, **16,** 352-358 (2008).
[26] Miller, J.C.; Holmes, M.C.; Wang, J.; Guschin, D.Y.; Lee, Y.-L.; Rupniewski, I.; Beausejour, Ch.M.; Waite, A.J.; Wang, N.S.; Kim, K.A.; Gregory, Ph.D.; Pabo, C.O.; Rebar, E.J., An improved zinc-finger nuclease architecture for highly specific genome editing. *Nature Biotechnol.* **25,** 778 (2007).
[27] Szczepek, M.; Brondani, V.; Buchel J.; Serrano, L.; Segal, D.J.; Cathomen, T., Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nature Biotechnol.* **25,** 786 (2007).