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Message from the Director

Founded in 1975 as an All –union Research Institute of Applied Enzymology, the present day Institute of Biotechnology is rapidly changing to meet the research and training needs of our technological society.

IBT has made great strides in the last several years with the aid of several European Union Structural Grants to improve the Institute's physical and personnel resources to better assist both faculty and industry in translating laboratory discoveries in these modern biology areas:

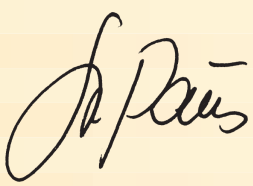
- Genetic and molecular studies of restriction- modification phenomeon
- Function of genes in yeast
- Research and development of recombinant biomedical proteins

IBT's emphasis and strong commitment to excellence in research and development has also led to an increasing awareness of the Institute's competitiveness within the international community. Selected as a Centre of Excellence in 2002 the recognition paid tribute to the quality of research and allowed hosting of numerous researchers, graduates, and two new laboratories (Lab. of Bioinformatics and Lab. of Biothermodynamics and Drug Design) headed by former expatriates.

Besides contributing to the biotechnological research and development, the Institute works to educate the public through partnerships with local and international educational efforts. The Institute supports the pursuit of learning about the promise, achievements, and challenges of biotechnology.

Encouraged by its results and growing recognition, IBT continues to strive for excellence by consolidating and focusing its competitive strengths and core competencies in order to stimulate the development of high value strategic opportunities. I firmly believe that through the concerted and integrated efforts of the public and private sectors, a viable biotechnology industry can become a reality. Moreover, a thriving biotechnology sector would further contribute towards the region's committed transformation into a high value-added and knowledge-based economy.

Algimantas Pauliukonis, Ph.D., Dr. Habil.
Director of the Institute



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Restriction enzymes are proteins made in bacteria and cut DNA at specific sites. More than 3500 Type II restriction enzymes with nearly 250 differing specificities have been identified to date (see REBASE database). Each recognizes a particular DNA sequence that is four to eight base pairs in length and cleaves phopshodiester bonds in the presence of Mg^{2+} within or close to their recognition sites. Restriction endonucleases have now gained widespread application as indispensable tools for the *in vitro* manipulation and cloning of DNA. However, much less is known about how they achieve their specificity and DNA cleavage. At the *Laboratory of Protein-DNA Interactions* we focus our studies on molecular mechanisms by which restriction enzymes achieve their function.

Among the questions being asked are:

- How different specificities evolve within the same structural fold?
- How different folds are tailored to function as restriction enzymes?
- How do the sequence recognition and catalysis are coupled in the function of restriction enzymes?

Answers to these questions are being sought using X-ray crystal structure determination of restriction enzyme-DNA complexes, site-directed mutageneses and biochemical/biophysical studies to relate structure to function.

Structure and function of the metal-independent restriction enzyme

Among all restriction endonucleases known to date, BfiI is unique in cleaving DNA in the absence of metal ions. BfiI represents a different evolutionary lineage of restriction enzymes, as shown by its crystal structure at 1.9-A resolution.

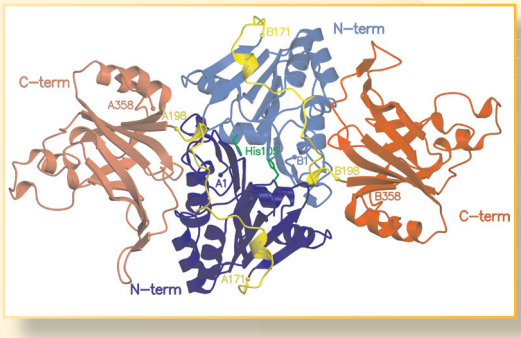

Figure 1. Crystal structure of BfiI dimer. Two N-term domains of each monomer depicted in light and dark blue, respectively, make a dimeric catalytic core flanked by C-term DNA-binding domains depicted in light and dark red. Linker connecting the N- and C-term domains within the monomer is shown in yellow.

The protein consists of two structural domains. The N-terminal catalytic domain is similar to Nuc, an EDTA-resistant nuclease from the phospholipase D superfamily. The C-terminal DNA-binding domain of BfiI exhibits a β-barrel-like structure very similar to the effector DNA-binding domain of the Mg^{2+}-dependent restriction enzyme EcoRII and to the B3-like DNA-binding domain of plant transcription factors. BfiI presumably evolved through domain fusion of a DNA-recognition element to a nonspecific nuclease akin to Nuc and elaborated a mechanism to limit DNA cleavage to a single double-strand break near the specific recognition sequence.

Reference: Grazulis S., Manakova E., Roessle M., Bochtler M., Tamulaitiene G., Huber R., Siksnys, V. Structure of the metal-independent restriction enzyme BfiI reveals fusion of a specific DNA binding domain with a non-specific nuclease. Proc Natl Acad Sci USA, 2005, 102,15797-15802

Principles of activity regulation of restriction enzymes

Restriction enzymes recognize short nucleotide sequences and cleave phosphodiester bond within or close to the recognition sequence. In bacteria cells bearing a restriction-modification system, host DNA is protected from the cleavage by methylation of the sequences recognized by restriction enzyme. Faithful sequence recognition by restriction endonuclease is crucial for the cell survival. To prevent cleavage at undesirable sites, the nuclease activity of restriction enzymes has to be tightly controlled and coupled to a particular DNA sequence. We use a combination of biochemical, mutational and structural methods to analyze the molecular mechanisms underlying cleavage activity control in restriction enzymes belonging to the three different subtypes.

Type IIF enzymes. Type IIF restriction enzymes are usually tetramers arranged as two homodimers. The Bse634I tetramer is composed of two primary dimers that resemble orthodox homodimeric restriction endonucleases. Two primary dimers are positioned back-to-back to each other with DNA binding clefts facing opposite directions. We have shown recently that a sin-

gle mutation W228A at the dimer-dimer interface converts tetrameric wt Bse634I into a dimer. This finding provided a unique opportunity to compare functional properties of the tetrameric and dimeric forms of the same restriction enzyme. We have shown that the cleavage activity of the primary dimer within the tetramer depends on the status of its partner dimer.

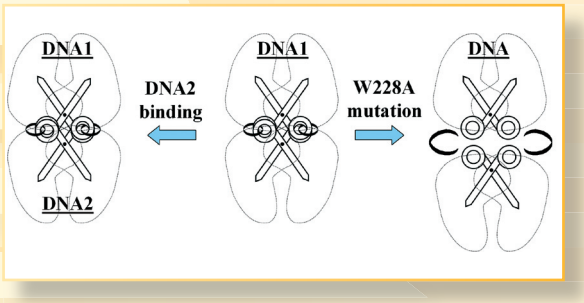

Figure 2. A scheme illustrating the communication between the dimers in the tetrameric Bse634I.

First, if one of the primary dimers is DNA-free, the catalytic activity of the DNA-bound dimer is reduced to a low residual level. In other words, the DNA-free dimer autoinhibits the cleavage activity of the DNA-bound partner, therefore, Bse634I is slow on the one-site DNA. Disruption of the inter-dimer interface by W228A mutation relieves the autoinhibition and unmasks the "true" inherent catalytic activity of the dimer. Alternatively, formation of the synaptic complex by binding of the second DNA copy by the partner dimer, eliminates the autoinhibition and tetramer cleaves both DNA sites at the same rate as dimeric W228A mutant.

Reference: Zaremba M., Sasnauskas G., Urbanke C., Siksnys, V. Conversion of the tetrameric restriction endonuclease Bse634I into a dimer: oligomeric structure-stability-function correlations. J Mol Biol., 2005, vol. 348, 459-78.

Type IIE enzymes.According to the current paradigm Type IIE restriction endonucleases are homodimeric proteins that simultaneously bind to two recognition sites but cleave DNA at only one site *per* turnover: the other site acts as an allosteric locus, activating the enzyme to cleave DNA at the first. Structural and biochemical analysis of the archetypal Type IIE restriction enzyme EcoRII suggests that it has three possible DNA binding interfaces enabling simultaneous binding of three recognitions sites. To test if putative synapsis of three binding sites has any functional significance, we have studied EcoRII cleavage of plasmids containing a single, two and three recognition sites under both single turnover and steady state conditions.

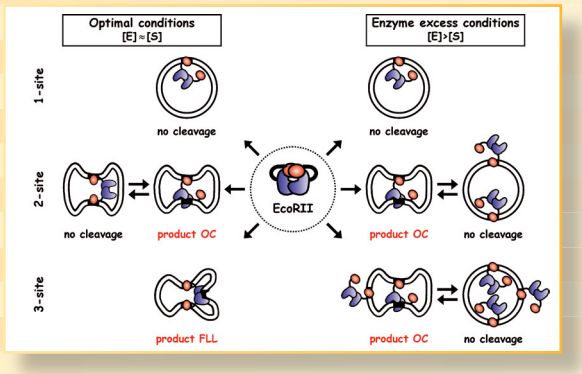

Figure 3. Schematic illustration of EcoRII interactions with 1-, 2- and 3-site plasmids under different experimental conditions. Possible EcoRII complexes formed on the 1-, 2- and 3-site plasmids under the *optimal* ([E]≈[S]) or *enzyme excess* ([E]>[S]) conditions are depicted. The N-terminal domain of EcoRII is shown in red and C-domain in blue, black rectangles in plasmids represent the EcoRII recognition sites.

We have shown that EcoRII displays distinct reaction patterns on different substrates: i) it shows virtually no activity on a single site plasmid, ii) it yields open-circular DNA form nicked at one strand as an obligatory intermediate acting on a two-site plasmid, iii) it cleaves concertedly both DNA strands at a single site during a single turnover on a three site plasmid to yield linear DNA. Taken together the data indicate that EcoRII requires simultaneous binding of three rather than two recognition sites *in cis* to achieve concerted DNA cleavage at a single site. We propose that other Type IIE restriction enzymes may employ the mechanism suggested here for EcoRII.

References:

1. Tamulaitis G., Mucke M., Siksnys V. Biochemical and mutational analysis of EcoRII functional domains reveals evolutionary links between restriction enzymes. *FEBS Letters*, submitted.
2. Tamulaitis G., Sasnauskas G., Mucke M., Siksnys V. Simultaneous binding of three recognition sites is necessary for a concerted plasmid DNA cleavage by EcoRII restriction endonuclease. *J.Mol.Biol.*, submitted.

Type IIS enzymes. The crystal structure of BfiI (Fig. 1) revealed that the protein consists of two structural domains. The N-terminal catalytic domain makes a dimer that is similar to Nuc, an EDTA-resistant nuclease from phospholipase D superfamily. The C-terminal domain of BfiI exhibits a β-barrel-like structure strikingly similar to the allosteric DNA-binding domain of the EcoRII. The crystal structure suggests that the interdomain linker may act as an autoinhibitor controlling BfiI catalytic activity in the absence of a specific DNA sequence. The linker connecting N- and C-terminal domains of the BfiI runs across the putative DNA binding surface at the N terminal domain of BfiI and sterically blocks DNA access to the active site buried at the interface between the two domains.

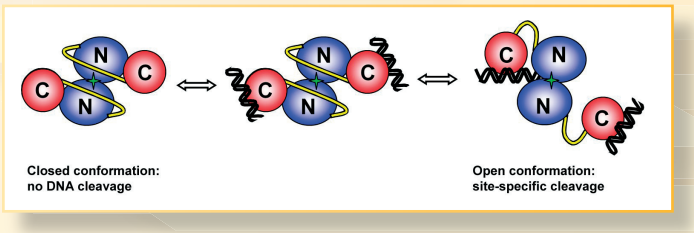

Figure 4. Possible model for BfiI catalytic activity control.

The BfiI enzyme in the absence of DNA is in a closed conformation (Fig. 4, left panel). Linkers connecting the N- and C-terminal domains run across the putative DNA binding interface at the catalytic domain and hinder the DNA binding. Specific DNA binding to the C-terminal domains (Fig. 4, middle panel) presumably triggers a conformational rearrangement that opens the active site at the catalytic domain and promotes double-strand break at specific site (Fig. 4, right panel). We use a combination of biochemical, mutational and structural methods to analyze if autoinhibition by the polypeptide linker plays a major role in the cleavage activity control in BfiI.

Structure-function-specificity relationships within subfamilies of restriction enzymes

Structural and bioinformatic data indicate that restriction endonucleases can be subdivided into distinct evolutionarily related families. In order to understand the structural basis of specificity evolution within the family members we have focused on the elucidation of the crystal structures of enzymes belonging to the two different subfamilies.

NgoMIV/Bse634I restriction endonuclease family. Restriction enzymes belonging to this family recognize related nucleotide sequences containing conserved CCGG or CCNGG subsequences that are flanked by different nucleotides (see, Table I).

Table I. Evolutionary conserved family of NgoMIV/Bse634I restriction enzymes: names, subtypes, recognition sequences, oligomeric structures

Enzyme	Subtype	Recognition sequence	Oligomeric state	Current status
HpyRFL100	?	/CCGG	?	Construction of the overproducing strain is going in the lab
Ecl18kI	IIP?	/CCNGG	Dimer?	Co-crystal structure solved in collaboration with M.Bochtler
EcoRII	IIE	/CCWGG	Dimer	Apo-structure published by Zhou, University of Alabama
Kpn2I	IIP	T/CCGGA	?	Diffracting crystals obtained in the lab
NgoMIV	IIF	G/CCGGC	Tetramer	Co-crystal structure published from the lab
Bse634I/Cfr10I	IIF	R/CCGGY	Tetramer	Apo-structures published from the lab
AgeI	IIP?	A/CCGGT	?	Overproducing strain obtained from NEB
PfoI	IIP	T/CCNGGA	?	Strain obtained from Fermentas, cloning is necessary
SgrAI	IIF	CR/CCGGYG	Dimer/ Tetramer	Crystals obtained by N.Horton at University of Arizona

The family currently includes 10 enzymes specific for the CCGG, CCNGG, CCWGG, NCCGGN, CRCCGGYG sequences spanning from 4 nt to 8 nt**.** The cleavage position within all these sequences is conserved (before the first C within conserved CC). Collectively, the enzymes belonging to this family represent nine different specificities (Table I). The family members presumably have a conserved structural core of the monomer, however, they show variability in sequence specificity and quaternary structure. Structural studies of enzymes belonging to this family should allow us to understand how different specificities emerge

within a common structural core. In parallel, understanding the oligomeric structure–function relationships within the family of the homologous restriction enzymes can provide clues to the biological and functional significance of different quaternary associations. Structural studies should reveal how Nature engineered different specificities within a conserved structural fold. Understanding of Nature's strategy should promote the design of new specificities by protein engineering.

SdaI/BsuBI/PstI restriction endonuclease family. SdaI protein sequence reveals significant similarities to BsuBI/PstI protein family, which includes the C-terminus of 9 proteins similar to Type IIP restriction enzymes BsuBI/PstI, specific for nucleotide sequence CTGCAG. Hexanucleotide recognition sequence of BsuBI/PstI overlaps with the central part of the SdaI target site CCTGCAGG. Both SdaI and BsuBI/PstI cleave their target sites after A base to produce 4 bp 3′ sticky ends. Protein sequence alignment between SdaI and seeding family members BsuBI/PstI reveals that ~30% identical and ~50% similar amino acids are conserved. The degree of sequence conservation between SdaI and other family members suggests possible structural similarities. We have solved the crystal structure of SdaI restriction enzyme providing a first structural template for the family.

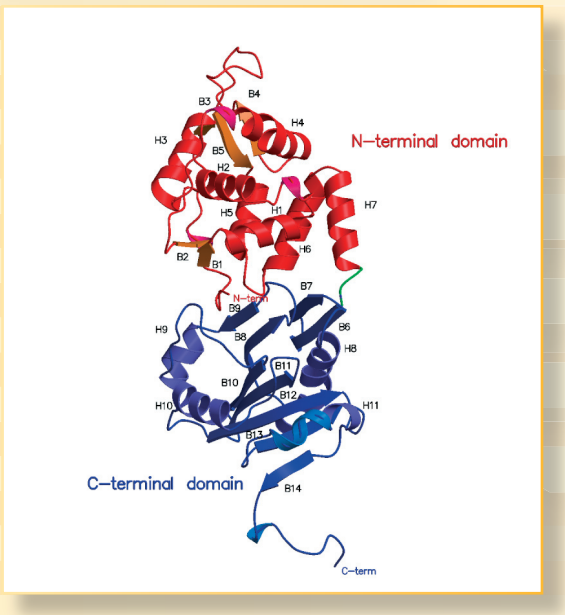

Figure 5. Crystal structure of SdaI restriction enzyme.

A PSI-BLAST search provides 22 protein sequences significantly similar to SdaI. However, only BsuBI/PstI and SbfI are known to be functional restriction enzymes. Other proteins are or may be annotated as putative restriction endonucleases due to the sequence similarity to BsuBI/PstI. Crystallization of the BsuBI is in progress.

Reference:

Tamulaitiene G., Jakubauskas A., Huber R., Grazulis S., Siksnys V. Crystal structure of SdaI restriction endonuclease reveals novel domain architecture (manuscript in preparation).

Novel folds of restriction enzymes

Crystal structure solution of BfiI revelead for the first time that different folds can be employed by restriction enzymes and shifted the paradigm of monophyletic origin of restriction endonucleases. Indeed, bioinformatic data supported by mutational and biochemical analysis suggest that restriction endonucleases comprise polyphyletic group of proteins that belong to the PD…(E/D)XK-, HNH-, PLD-, GIY-YIG families. However, except of BfiI we still lack structural evidence how different folds are adapted to function as restriction enzymes. We have started the project determining the structural organization and the mode of action of the GIY-YIG family of restriction endonucleases Ecl29I/Cfr42I using a combination of mutational analysis, biochemical techniques, kinetic analysis and X-ray crystallography.

The vast majority of TypeII REases introduce double strand breaks in DNA at fixed location within or close to their recognition sites. Restriction endonuclease BpuJI specific for the CCCGT sequence (J.Vitkute, unpublished data) instead of producing a fixed cleavage pattern characteristic for most of restriction enzymes, generates multiple variable cuts located outside of the

recognition site. We lack any biochemical or structural data to correlate the variable cleavage pattern with enzyme's structural organization. We have started the project determining the structural organization and the mode of action of BpuJI using a combination of mutational analysis, biochemical techniques, kinetic analysis and X-ray crystallography.

Grants

Howard Hughes Medical Institute, International Research Scholars Program
Wellcome Trust
International Quality Network; DAAD, Germany
EC Fifth Framework Programme (FP5)
Lithuanian Science and Studies Foundation
High-Technology Development Programme

Collaborations

Dr. Matthias Bochtler, International Institute of Molecular and Cell Biology
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Prof. Dr. S.E.Halford, Bristol University
Prof. Dr. Robert Huber, MPI of Biochemistry, Martinsried
Prof. Dr. Alfred Pingoud, University of Giessen
Dr. Monika Reuter, Institute of Virology, Berlin
Dr. A. Solonin, Institute of Biochemistry and Physiology of Microorganisms, Moscow
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Publications 2004-2005

1. Tamulaitis G., Sasnauskas G., Mucke M., Siksnys V. Simultaneous binding of three recognition sites is necessary for a concerted plasmid DNA cleavage by EcoRII restriction endonuclease. J.Mol.Biol., submitted.
2. Tamulaitis G., Mucke M., Siksnys V. Biochemical and mutational analysis of EcoRII functional domains reveals evolutionary links between restriction enzymes. FEBS Letters, submitted.
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6. Zaremba M., Urbanke C., Halford S.E., Siksnys V. Generation of the BfiI restriction endonuclease from the fusion of a DNA recognition domain to a non-specific nuclease from the phospholipase D superfamily. J Mol Biol., 2004, vol. 336, 81-92.

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Laboratory of Biological DNA Modification

Enzymatic methylation of nucleobases serves to expand the information content of the genome in organisms ranging from bacteria to mammals. Postreplicative methylation is accomplished by DNA methyltransferases, which catalyze the transfer of a methyl group from cofactor S-adenosyl-l-methionine (AdoMet) yielding 5-methyl cytosine, N4-methylcytosine or N6-methyladenine. DNA methylation is involved in the control of transcription, genomic imprinting, and developmental regulation in eukaryotes. Aberrations in cytosine-5 methylation play a role in human genetic disease, and, therefore, the MTases are potent candidate targets for anticancer therapy. MTases are obligatory enzyme constituents of restriction-modification systems found in most bacterial organisms. DNA cytosine-5 MTases from both eukaryotes and prokaryotes comprise a family of homologous enzymes.

Besides their diverse biological roles DNA methyltransferases (MTases) are attractive models for studying structural aspects of DNA-protein interaction. Bacterial enzymes recognize an impressive variety (over 200) of short sequences in DNA. As shown first for the HhaI MTase, access to the target base that is buried within the stacked double helix is gained in a remarkably elegant manner: by rotating the nucleotide completely out of the DNA helix and into a concave catalytic pocket in the enzyme. This general mechanistic feature named "base-flipping" is shared by numerous other DNA repair and DNA modifying enzymes. Our laboratory studies the mechanistic and structural aspects of DNA methylation using the HhaI methyltransferase (M.HhaI) from the bacterium *Haemophilus haemolyticus* as the paradigm model system.

Kinetic and molecular mechanism of DNA methylation

Enzymatic DNA cytosine-5 methylation is a complex reaction which proceeds via multiple steps such as binding of cofactor AdoMet and substrate DNA, flipping of the target cytosine, conformational rearrangement of the mobile catalytic loop, activation of the target cytosine via formation of a transient covalent bond, methyl transfer and so on. We use mutagenesis, biochemical analysis, steady-state and transient kinetic analysis, fluorescence spectroscopy and x-ray diffraction to delineate the elementary steps on the reaction pathway of HhaI MTase.

To elucidate the role of cofactor interactions during catalysis, eight mutants of the Trp41 residue, which is located in the cofactor binding pocket, were constructed and characterized. The mutants show full proficiency in DNA binding and base flipping, and little variation is observed in the apparent methyl transfer rate. However, the Trp41 replacements with short side chains substantially perturb cofactor binding leading to faster (up to ten-fold) turnover of the enzyme. Our analysis shows that the breakdown of a long-lived ternary product complex is initiated by the dissociation of AdoHcy or the opening of the catalytic loop in the enzyme, which for the first time identifies rate-limiting events in the catalytic cycle of a DNA cytosine-5 methyltransferase (3).

Rotation of a nucleotide out of the DNA helix (base flipping) is a mechanistic feature used by numerous DNA modification and repair enzymes. Despite of extensive studies of the HhaI cytosine-5 methyltransferase, initial events in the base flipping mechanism of this model enzyme remained elusive. We found that the replacement of the target C:G pair with the 2-aminopurine:T pair in the DNA or certain mutations of Gln237 in the protein severely perturb base flipping, but retain specific DNA binding. Kinetic analyses and molecular modeling suggest that a steric interaction between the protruding side chain of Gln237 and the target cytosine in B-DNA reduces the energy barrier for flipping by 3 kcal/mol. Subsequent stabilization of an open state by further 4 kcal/mol is achieved through specific hydrogen bonding of the side chain to the orphan guanine. Deletion mutations in the catalytic loop have no effect on DNA binding and flipping of the 2-aminopurine base. Gln237 thus plays a key role in actively opening the target C:G pair by a "push-and-bind" mechanism. This finding illustrates a "strain" mechanism in DNA enzymes, which serves to utilize substrate binding energy in catalysis (1).

To obtain further insights into dynamics of the base flipping we have initiated an NMR structural study of the dynamics of the HhaI MTase-DNA system in solution (in collaboration with Prof. Thomas Szyperski, SUNY Buffalo, NY, USA). We have recently succeeded in engineered functional variants of the enzyme that show a significantly increased solubility and long term stability. This now paves the way to detailed structural studies of this model cytosine-5 MTase and its interactions with the ligands employing NMR spectroscopy.

DNA base flipping is an important mechanism in molecular enzymology, but its study is limited by the lack of an accessible and reliable diagnostic technique. A series of crystalline complexes of a DNA methyltransferase, M.HhaI, and its cognate DNA, in which a fluorescent nucleobase analogue, 2-aminopurine, occupies defined positions with respect the target flipped base, have been prepared and their structures determined at higher than 2Å resolution. From time-resolved fluorescence measurements of these single crystals, we have established that the fluorescence decay function of 2-aminopurine shows a pronoun-

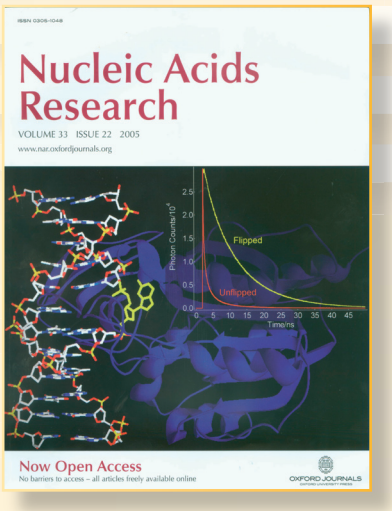

Figure 1. Journal cover illustration highlighting the structure and time-resolved fluorescence decay profiles of crystalline M.HhaI-DNA complexes containing the fluorescent DNA base analog 2-aminopurine.

ced, characteristic response to base flipping: the loss of the very short (~100 ps) decay component and the large increase in the amplitude of the long (~10 ns) component. When 2-aminopurine is positioned at sites other than the target site, this response is not seen. Most significantly, we have shown that the same clear response is apparent when M.HhaI complexes with DNA in solution, giving an unambiguous signal of base flipping. Analysis of the 2-aminopurine fluorescence decay function reveals conformational heterogeneity in the DNA-enzyme complexes that cannot be discerned from the present x-ray structures (4).

Structure-function analysis of mammalian DNA methyltransferases

In higher eukaryotes including mammals, DNA methylation plays a key role in the control of numerous cellular processes such as transcription, genomic imprinting, developmental regulation. Three major types of MTases are known in mammals, which are large multidomain proteins involved in intricate interactions with other cellular components. We study the mechanism and interactions of Dnmt1 and Dnmt3a,b MTases using mutagenesis, chemical foot-printing, kinetic analysis and fluorescence spectroscopy (collaboration with Prof. S. Tajima, Osaka University, Japan). DNA methyltransferase Dnmt1 ensures clonal transmission of lineage-specific DNA methylation patterns in a mammalian genome during replication. To understand the underlying mechanism of its maintenance function, we purified recombinant forms of full length Dnmt1, a truncated form of Dnmt1 (291-1620) lacking the binding sites for PCNA and DNA, and examined their processivity, using a series of long unmethylated and hemimethylated DNA substrates. Direct analysis of methylation patterns using bisulfite-sequencing and hairpin-PCR techniques demonstrated that full length Dnmt1 methylates hemimethylated DNA with high processivity and a fidelity. The truncated form of Dnmt1 showed identical properties to full length Dnmt1 indicating that the N-terminal 290 amino acid residue region of Dnmt1 is not required for the preferential activity towards hemimethylated sites or the processivity of the enzyme. Remarkably, our analyses also revealed that Dnmt1 methylates hemimethylated CpG sites on one strand of double-stranded DNA during a single processive run. These findings suggest that the inherent enzymatic properties of Dnmt1 play an essential role in the faithful and efficient maintenance of methylation patterns in mammalian genome (2).

Engineering Methyltransferases for targeted covalent modification of DNA

The goal of this study is to develop new molecular tools for sequence-specific covalent modification of DNA. Using the HhaI MTase as a model system, we are exploring two approaches. One strategy is to redesign the catalytic site of M.HhaI such that the target cytosine base would be excised rather than methylated thereby creating a new enzymatic activity – a sequence-specific cytosine DNA glycosylase. Rational protein design and directed evolution are employed to produce Mtase variants endowed with the new activity (in collaboration with Dr. Janusz Bujnicki, International Institute of Molecular and Cell Biology, Warsaw, Poland). Currently, analysis of the obtained variants is underway to assess their catalytic efficiency in vitro.

The other approach, which is based on synthetic analogues of the natural cofactor AdoMet, has led to a significant breakthrough in the sequence-specific covalent modification of DNA (collaboration with Prof. Elmar Weinhold, Institute of Organic Chemistry, RWTH Aachen, Germany). We chemically synthesized a series of AdoMet analogs with extended methyl replacements carrying in addition to alkyl groups, alkenyl and alkynyl groups by alkylation of *S*-adenosyl-L-homocysteine. The synthe-

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sized AdoMet analogs were tested with representatives of all three classes of DNA Mtases. Consistent with previous observations, we detect no or almost no transfer of the propyl group under various reactions conditions. Remarkably, the AdoMet analogs with methyl group replacements containing a carbon-carbon double (allylic system) or triple bond (propargylic system) in β position to the sulfonium center proved much more efficient. These cofactors render full modification of long natural DNA at sub-stoichiometric amounts of MTases over their recognition sites, demonstrating that the poor reactivity of AdoMet analogs with saturated groups can be overcome by placing an activating group (unsaturated C-C bond) next to the carbon unit that is attacked. Enzymatic transmethylation reactions are thought to proceed via an S_N2 mechanism with inversion of configuration at the transferred methyl group. The chemical rationale for such reactivation apparently derives from conjugative stabilization of the *p* orbital at the reactive carbon formed during the transition state. The enzymatic transalkylations of DNA occurred in a highly sequence-specific manner. Notably, removal of the essential catalytic cysteine residue in M.HhaI (C81S mutation) completely abolishes the transalkylation reactions, confirming that the transfer of the extended groups proceeds via the natural catalytic mechanism. The MTase-mediated transalkylations are thus truly catalytic, i.e. the enzyme is released after the reaction for subsequent turnovers. The transalkylation rates of up to 30 turnovers per hour indicate that the sequence-specific derivatization of DNA is a convenient technique suitable for routine laboratory use (5, 6).

The aforementioned results demonstrate that the AdoMet analogs with extended allylic and propargylic groups function as efficient cofactors for DNA MTases, which provides a completely new powerful technique for sequence-specific covalent derivatization of DNA (reviewed in Breindl, A. *BioWorld Today* 2005 16:1; Borman, S. *Chem. Eng. News* 2005, 83: 12; Mercer, A.C. & Burkart, M.D. *Nature Chem. Biol.* 2006, 2: 8-11). As shown for restriction endonucleases, bulky groups can be deposited at

Giedrius Vilkaitis, Ph. D.

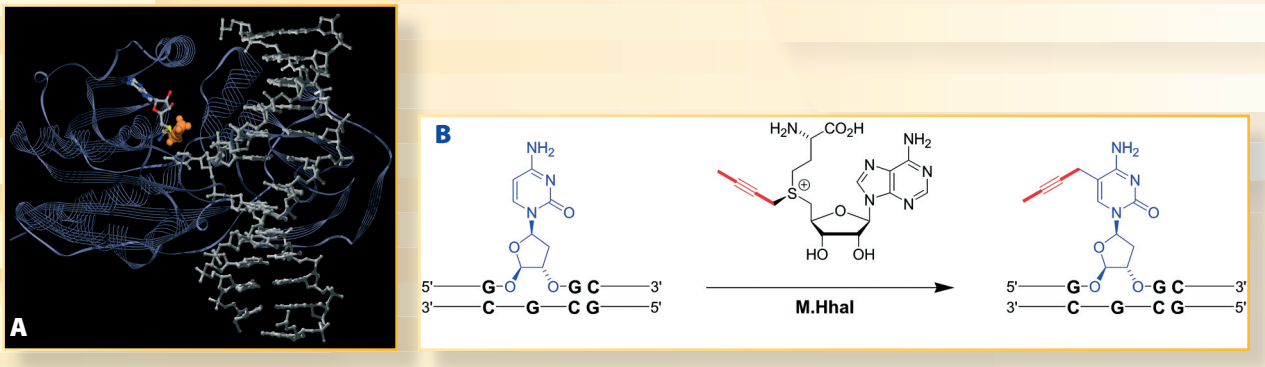

Figure 2. Rapid transit: left (A), a model of the butynyl AdoMet analog bound to the HhaI methyltransferase (blue ribbon structure).The analog's extended group (orange) is about to be transferred to DNA (gray); below (B), a scheme for the enzymatic transfer of the butynyl group (red) from the synthetic cofactor onto the target cytosine (blue) within the GCGC site in DNA.

specific sites to interfere with the action of DNA-modifying enzymes or DNA-binding proteins. Furthermore, functional groups could be appended to the side chains of the cofactors, sequence-specifically transferred to DNA and then modified with chemical entities in chemo-selective ligation reactions. Alternatively, a desired label could be directly attached to the side chain of the allylic and propargylic AdoMet analogs and sequence-specifically transferred to DNA. The REBASE database currently lists about 800 DNA MTases that recognize over 200 different DNA recognition sequences spanning 2–8 base pairs, offering an unprecedented experimental control over sequence-specific manipulation of DNA with many potential applications ranging from probes for genetic screening technologies to molecular building blocks in DNA-based nanobiotechnology. Moreover, the newly developed cofactors should in principle be suitable for sequence-specific transfer of functional groups or other chemical entities to RNA and proteins using appropriate MTases as catalysts.

Grants

Howard Hughes Medical Institute, International Research Scholars Program
NATO Science Programme, Collaborative Linkage Grant
Royal Society, Joint Projects under European Science Exchange Programme
International Bureau of BMBF, Zusammenarbeit mit den Mittel- und Osteuropäischen Ländern (Germany)
Lithuanian Science and Studies Foundation

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Patents

Weinhold E., Dalhoff C., Klimašauskas S., Lukinavičius G. (2005) "New S-adenosyl-L-methionine analogues with extended activated groups for transfer by methyltransferases", European patent application No. 05008226.2

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Introduction

Research in our laboratory is concentrated mainly toward the investigation of different aspects of the restriction-modification (RM) phenomenon, with emphasis on the practical applications of scientific results. We are focused on the sequence-structure-function relationships of the R-M components: restriction endonucleases (REases) and DNA methyltransferases. To date, numerous attempts have been made worldwide to understand the mode of DNA recognition and to change the specificity of REases, the enzymes of high commercial value. This resulted in more than 15 high-quality X-ray structures of different REases. Nevertheless, information gained in this way appeared to be insufficient to rationally engineer restriction enzymes with new specificities. Recently, we have developed a technology called methylation activity-based selection for the isolation of REases with novel specificities. This technology allowed us to isolate, for the first time in the world, the REase *Eco*57MI with a new specificity. Our laboratory is heading toward creating new instruments for DNA manipulation using approaches of rational design and directed molecular evolution.

Investigation of type II bifunctional restriction endonucleases

Previously, we have demonstrated that AloI RM and PpiI RM systems recognize DNA targets of similar structure (↓7/12-13(N)**GAAC**$(N)_6$**TCC**(N)12-13/7↓ and ↓7/12(N)**GAAC**$(N)_5$**CTC**(N)13/8↓ respectively) and cleave DNA in very similar fashion (as indicated by arrows). They comprise only one large protein which *in vivo* and *in vitro* performs both DNA restriction and modification. Basing on comparative analysis and gene mutagenesis experiments we have shown that the primary structures of both REases are also similar: N-terminal region is responsible for the DNA cleavage, the central part - for DNA methylation, and C-terminus - for DNA target recognition. Domain swapping experiments between AloI and PpiI clearly indicated that C-terminal parts of enzymes are interchangeable and that they are responsible for the target recognition. Also, we found that highly similar AloI and PpiI regions (TRD2), which we predicted to be responsible for the recognition of common tetranucleotide GAAC, are interchangeable as well and their replacement could result in mutants of wild-type specificity. In contrast, exchange of protein regions (TRD1) putatively responsible for the recognition of trinucleotide of complex DNA targets resulted in specificity of hybrids which was the same as the specificity of enzyme which have donated corresponding protein region. We concluded, that genes of AloI and PpiI REases and other type IIB restriction enzymes are very good source to construct hybrid restriction endonucleases with predicted sequence specificities. We cloned third member of type IIB restriction endonuclease TstI recognizing ↓13/8(N)CAC$(N)_6$TCC(N)12/7↓ DNA target. As we predicted, TstI has similar primary structure as AloI or PpiI and contains potential TRD's in C-terminus of polypeptide. One of them, named VR1, was expected to be responsible for recognition of TCC trinucleotide. This presumption has been tested by replacing mentioned region by TRD1 domain from PpiI restriction endonuclease. New hybrid enzyme is active and recognizes DNA targets 5'-CACN_5CTC (manuscript in preparation).

As have been mentioned in previous report, our laboratory developed a new procedure called the Methylation Activity Based Selection (MABS) for generating REases with new sequence specificity. MABS uses a property of bifunctional type II REases to methylate DNA targets they recognize. The efficiency of this technique was demonstrated by altering target specificity of the bifunctional REase Eco57I. We have used this technique in order to select mutants with altered DNA sequence specificity of another bifunctional enzyme AloI. *aloI* gene region encoding TRD1 was mutated *in vitro* and MABS technology was exploited to select AloI variants which are able to modify and partially cleave 5'-GAACN_6TNC targets. Currently, we are selecting new AloI variants with higher catalytic efficiencies.

Investigation of type IIS restriction endonucleases

Type IIS restriction endonucleases recognize asymmetric targets and cleave both DNA strands outside the target. Only one enzyme of this group, FokI, was analyzed in details. Proteolytic and X-ray studies have indicated that FokI is composed of two domains, one of them being responsible for DNA recognition and the other one – for cleavage. In order to test if other representatives of this group have similar structural organization, we cloned, sequenced and investigated several type IIS restriction-modification systems.

The MnlI restriction endonuclease recognizes CCTC targets and cleaves upper DNA strand 7 bp and bottom strand 6 bp to the right. The C-terminal part of cloned and sequenced MnlI restriction endonuclease revealed intriguing similarity with

the nuclease domain of "H-N-H" type of bacterial toxins, Colicin E7 and Colicin E9, and allowed us to identify the conserved sequence motif $Rx_3ExHHx_{14}Nx_8H$ of MnlI as a new putative catalytic site in the family of restriction enzymes. We observed that mutations of conserved amino acids have only minor effect on the specific DNA binding but can cause sharp reduction or even total inactivation of catalytic activity. Moreover, MnlI cleaves both double stranded and single stranded DNA in the presence of magnesium and nickel ions, respectively, like abovementioned Colicines. These results indicate that MnlI differs from FokI, the member of so-called "PD-EXK" super family, and BfiI, the recently described member of phospholipase D superfamily, by both the active site architecture and the reaction mechanism, and in fact represents a new subtype of type IIS restriction endonucleases. Through the past two years oligomeric state and stoichiometry of MnlI-DNA complexes were detected. Gel-filtration assays have revealed, that R.MnlI forms a dimer in solution and thereby differs from other type IIS REases. By DNA gel-mobility shift assays we demonstrated, that R.MnlI can bind one or two DNA molecules, depending on conditions. We mapped the domain structure of R.MnlI using limited proteolysis technique. Preliminary data indicate that MnlI REase polypeptide forms two structural domains, one responsible for DNA recognition and another one for nucleolytic function. Experiments which should help to prove or deny the Colicin-related functions of nucleolytic domain are under way.

Another interesting feature performed by MnlI REase was observed - it is able to repress its own gene expression in *E.coli* host. Whether this is true for native enviromental conditions (*Moraxella nonliquefaciens*) still remains unproved.

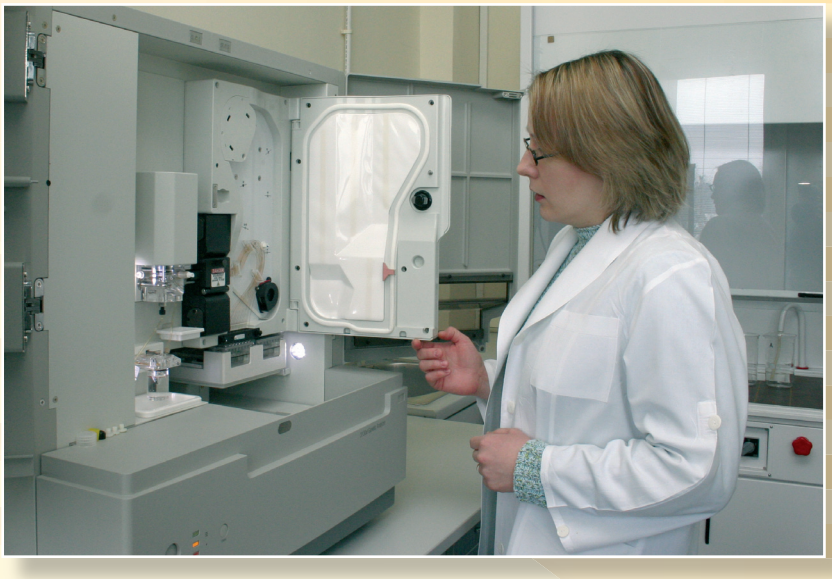
In an ongoing search for evolutionary related REases that recognise partially overlapping target sites, we have cloned and sequenced genes of three Type IIS RM systems, Alw26I (GTCTC), Eco31I (GGTCTC) and Esp3I (CGTCTC), which target sites share the common pentanucleotide, GTCTC. Our study showed that the deduced aa sequences of the Alw26I, Eco31I and Esp3I REases share significant level of identity. In a pairwise comparison, the amino acid identity score is higher than that observed hitherto among the REases which recognise partially overlapping nucleotide sequences. With this information in hand, Alw26I, Eco31I and Esp3I REases represent an exceptionally attractive group of proteins to study structure-function relationships in DNA recognition. In addition, the accompanying methyltransferases of the respective restriction-modification systems, are found to be represented by proteins of an unusual structural and functional organisation. Eco31I has been investigated to determine the structural-functional organization. We mapped the domain structure of Eco31I using limited proteolysis technique. It has been shown that Eco31I, as well as the best understood Type IIS REase FokI, consists of two structural domains. DNA gel-mobility shift and gel-filtration assays were used to determine functional properties of obtained proteolytic domains. Obtained results revealed that the N-terminal domain is responsible for specific DNA binding, while the C-terminal domain does not interact with DNA. In addition, it was supposed that C-terminal domain might be necessary for interaction of two Eco31I molecules as in the case of FokI dimer formation. Using error-prone PCR mutagenesis we determined the amino acid residues involved in DNA restriction function. Analysis of obtained mutant proteins indicated that C-terminal domain is responsible for DNA restriction.

Testing essentiality of pathogenic microbacterium genes

After the first complete genome sequence was published (Fleischmann et al., 1995), there has been a proliferation of genome sequencing projects. With reference to Genome OnLine Database (http://www.genomesonline.org/) it could be stated that more than 343 genomes had been completely sequenced to date. Database includes 25 Archaeal 277 Bacterial and 41 Eukaryal genomes. The extraordinary level of activity taking place in this field is highlighted by the fact that more than 993 prokaryotic and eukaryotic genome projects are underway.

Bacterial genome sequences are typically published with a considerable level of bioinformatic analysis already in place. Transfer RNAs, ribosomal RNAs, repetitive elements, and likely ORFs are usually identified and annotated. Often a general comparative analysis of genome composition and gene families is included to highlight the primary differences in the biosynthetic and regulatory pathways of the organism in comparison with previously published genomes. Although bioinformatics is a powerful tool for formulating hypotheses about gene function, conservation of a gene product between multiple bacterial species is not a guarantee that the gene product is essential for growth. Similar gene products can perform different functions for the cell, and proteins of nearly completely different sequence can perform the same function. The presence of a gene says nothing about when or if it is expressed or how important it is in the biology of the organism.

Identification of essential genes that have no defined function provides a starting point for uncovering novel and important biological processes in microorganisms. This is especially important in knowledge of pathogenic bacteria since the use of antibiotics to treat a range of disease is increasingly being compromised by the rise of drug resistance. Multiply resistant bacterial



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species are now common in the clinic. Product of essential gene which is absolutely required for survival could serve as new antimicrobial enzyme targets for efficient vaccines or the chemical inhibition resulting in cell stasis or death of pathogen.

Our laboratory have idea how simply to investigate *H.pylori* J99 strain for which genome sequences are available, and identify complete set of essential genes. To identify essential genes of *H. pylori* J99 we are preparing new methodology, which, like other essential genes testing methodologies, involves creation of reliable mutant collection of bacterium strain of interest and characterization of every single gene that tolerate disruption. An identification of many regions susceptible for mutagenesis per one sequencing reaction is major advantage of the approach. At the presence, we have already prepared reliable mutant collection of *H.pylori* J99 strain and preparing final experiments for sequencing reactions.

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Combined immune and gene therapy of chronic viral hepatitis

This project was supported by the EC Framework 5 programme, project Nr. QLK2-CT-2000-01476. The project began 2001-2002.

Many infectious agents persist in the patient because of an insufficient immune response. Natural viral capsids are highly immunogenic but often they do not induce neutralizing antibodies or a CTL response. Using genetic methods protective B and T cell epitopes can be introduced into capsid proteins and expressed in bacteria or yeast. It is planned to encapsidate in vitro into such chimeric capsids genes encoding cytokines, which favor Th1 and subsequent CTL responses. Recognition of such capsids by B cells would lead to efficient antibody production and Th1 cell epitope presentation in a favorable cytokine milieu. The suitability of such hepatitis B and polyoma virus capsid vectors shall be tested in cell cultures, and in mouse models.

For the construction of immunogenic chimeric VLPs forming proteins, polyomaviruses MPyV and SV40 VP1 proteins were used as carriers of selected HBV epitopes. Polyomavirus virus-like particles (VLPs) can be generated by heterologous expression and spontaneous self-assembly of major surface protein VP1. Although non-infectious, VLPs resemble virions in their strong immunogenicity and can be used as safe and efficient vaccines. VLPs tolerate the insertion and surface exposure of foreign epitopes and potentiate their immunogenicity.

First of all, both SV40 and MPyV VP1s were expressed in the yeast *S. cerevisiae* in order to test the efficiency of VLPs formation in the yeast expression system. Results of expression confirmed, that VP1s of both viruses efficiently formed VLPs in yeast.

The yield of CsCl purified particles made up approximately 30-40mg / l liter of yeast culture.

For the construction of chimeric VLPs, four potential sites were selected in the structure of both viruses VP1 on the basis of three-dimensional structure of SV40 VP1. The selected sites were localized in the loops exposed to the surface of virus particle: Site 1 - BC-loop, 73-74 aa; Site 2 - EF-loop, 198-199 aa; Site 3 - F-G1-loop, 228-229 aa; Site 4 – HI-loop, 273-274 aa. The sites for the restriction endonuclease *Bgl*II were inserted in the selected position of the SV40 and MPyV VP1 genes by using specific primers and PCR.

For the construction of chimeric VLPs, three different HBV PreS and S sequences, encoding main B and T cell epitopes were selected and inserted into modified VP1 genes. Two PreS1 sequence variants encoding the most important PreS1 epitopes were selected. The first PreS1 sequence variant included PreS1-20-47aa fragment, the second variant included all PreS1 sequence except the hydrophobic part between 58 – 90 aa - 13-58//90-119 aa. The third variant was S sequence encoding main antigenic loop of the S protein (101-169aa), exposed to the surface of HBV particles.

HBV *ayw* serotipe, provided by Prof. P.Pumpens (Riga) was used in these experiments.

All three HBV sequences were inserted into four different SV40 VP1and MPyV VP1 positions. The resulted hybrid genes were inserted into yeast expression vector and expressed in the yeast *S. cerevisiae*. Expression level and VLPs formation were investigated. Chimeric proteins that formed sufficient yield of VLPs (6 derived from SV40 VP1 and 4 derived from MPyV VP1) were selected for further investigations. Chimeric proteins containing two different inserts in the different VP1 loops of the same protein molecule were constructed also. CsCl and EM analysis of such chimeric proteins revealed, that both SV40 VP1 and MPyV VP1 tolerated double large insertions. The chimeric SV40 and MPyV VLP's containing HBV S sequence (101-169 aa) inserted in the BC loop and PreS1 sequence (13-59/90-119) inserted in HI loop were constructed and purified.

The purified chimeric VLPs were provided to project partner Prof. P.Pumpens for the investigation of immunogenicity of chimeric particles. Imunization experiments were finished; the corresponding publication is under preparation.

The initial transfections experiments of model gene (GFP) demonstrated, that chimeric particles as well as wt VP1 were not efficient tools for gene delivery, in comparison to transfection agents like Exgene (Fermentas UAB, Vilnius).

The next part of this project was the construction and investigation of different PreS1 deletion variants. Previous study revealed that the construction PreS1 sequence (13-59/90-119) + HBV S efficiently formed particles in yeast. The construct which included PreS1 13-59 aa also formed particles very efficiently, however the construct which included only 90-119 aa, did not form particles. Deletion analysis revealed that the N-terminal part of PreS1 is responsible for efficient particles formation in yeast. All previous PreS1 containing constructions have changed the first aa Gly to Ser. The construction (13-59/90-119) + HBV S containing N-terminal Gly also formed particles efficiently. The N-terminal Gly serve for myristoylation in all eukaryote organisms. This construction appeared will be important, because myristoylated PreS1 +HBV S stimulate binding of recombinant protein to hepatocytes.

Experiment carried out at Institute of Virology, Giessen University revealed that infectivity of HBV virus could be blocked efficiently in Tupaia system by amino-terminally acylated peptides containing amino acids (aa) 2-18 from the preS1 domain.

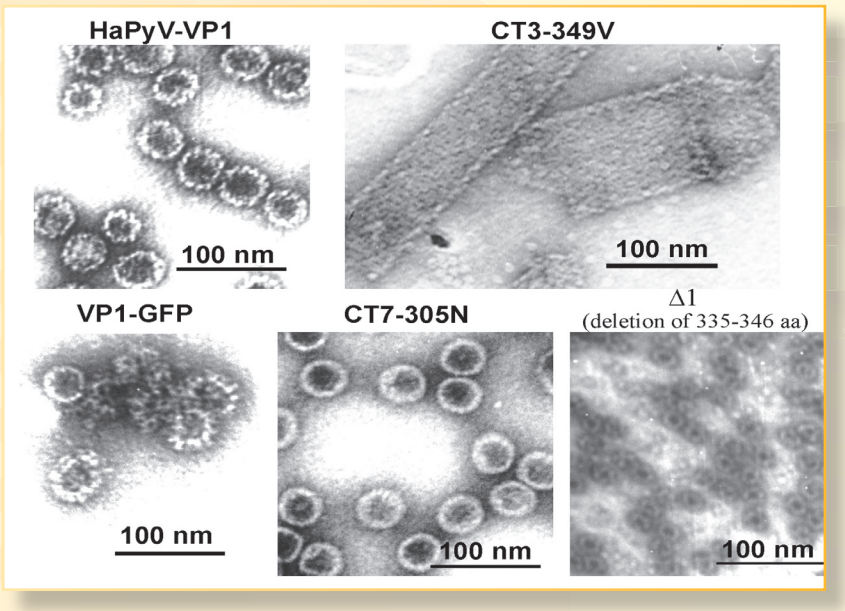
Addition of aa 28-48 enhanced the inhibitory capacity whereas aa 49-78 did not contribute to inhibition. Myristoylated preS1 peptide 2-48 bound strongly to Tupaia hepatocytes but not to non-hepatic cells or rodent hepatocytes and thereby inhibited infection even at concentrations of 1 nM completely. Particles consisting only of the small hepatitis B surface protein -the active component of current hepatitis B vaccines- did not bind at all to Tupaia hepatocytes but addition of the preS1 domain to the particles allowed binding. PreS1 sequence 2-48 mediates attachment of the virus to its target cells whereas the small surface protein seems to be involved in other steps. These findings implicate that the current subunit hepatitis B vaccines may be improved by addition of distinct preS1 epitopes. Immunisation experiments, carried out by Dr. A.Žvirblienė (The Laboratory of Immunology and Cell Biology) revealed that the immunogenicity of PreS1 containing HBV S proteins are significantly higher in comparison to the HBV S vaccine protein. Moreover, preS1 lipopeptides are promising candidates for specific antiviral therapy against hepatitis B infections. These results were published in the *Gastroenterology*.

Hamster polyomavirus-derived virus-like particles as a promising universal carrier for various vaccine development applications

Virus-like particles (VLPs) can be generated by heterologous expression of viral capsid and envelope proteins and their subsequent spontaneous self-assembly *in vivo* or *in vitro*. VLPs mimick infectious viruses in their structural and immunological features but are non-infectious and highly safe because of the lack of a viral genome and can therefore represent an ideal vaccine. VLPs can induce a strong humoral immune response because of correct folding of the monomeric proteins, the resulting formation of conformational antigenic determinants, the multimeric structure of identical subunits, and the presence of T_H epitopes. Moreover, VLPs are able to stimulate cytotoxic T cells. VLP-based vaccines are already in routine use for immunization against hepatitis B virus and were tested for their efficiency to protect against papilloma- and rotavirus infections in animal models.

Epitopes and receptor-binding ligands from proteins of pathogens, which are not able to form VLPs, can be grafted onto VLPs by genetic engineering to improve their immunogenicity and may profit from the highly organized structure of VLPs. The localization of potential insertion sites for foreign peptide sequences is essential for the generation of chimeric VLPs as well as evaluation of foreign peptide size limits that can be inserted. In fact, fusion of longer or multiple copies of peptides/ epitopes to VLPs usually introduces structural difficulties in protein folding and VLP formation.

The hamster polyomavirus (HaPyV) major capsid protein VP1 belongs to a family of polyomavirus VP1 proteins containing highly conserved structure motifs and functional domains. Yeast-expressed HaPyV-VP1 well tolerated the insertion of a foreign pentapeptide – an epitope with the sequence DPAFR originated from the preS1 region of HBV - at all four predicted surface-exposed sites between amino acid (aa) positions 80-89 (site #1), 222-225 (site #2), 243-247 (site #3) and 288-295 (site #4) of VP1. The insertion into VP1 did not influence protein folding and assembly of VLPs, and even allowed the generation of high-titered



VLP formation of HaPyV-VP1, its mutants and chimeric VP1-GFP protein after expression in yeast determined by negative staining electron microscopy.

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preS1-specific antibodies in mice. The high carrier competency of HaPyV-VP1 was also reflected by the fact that it tolerated not only single insertion of foreign sequences, but also the simultaneous insertions into different sites.

HaPyV-VP1 carrier was also studed in comparison to other carrier systems, in terms of insertion capacity, antigenicity and immunogenicity. Therefore we inserted the segments of Puumala hantavirus (strain Vranica/Hällnäs; PUUV-VR) nucleocapsid (N) protein containing 45, 80 or 120 N-terminal amino acid residues, previously used for insertion into HBV core-derived particles, into the four potential insertion sites of HaPyV-VP1. HaPyV-VP1 well tolerated the insertion of VR peptides at surface-exposed sites between aa positions 80 – 89 (site #1) or 288 – 295 (site #4) without influencing protein folding and assembly of VLPs. Site #4 of HaPyV-VP1 seemed to tolerate even the insertion of green fluorescent protein. The insertion capacity at sites #2 (between aa 222-225) and #3 (between aa 243-247) was limited; insertions of short peptides were tolerated but longer foreign peptides derived from PUUV-VR N protein apparently affected correct folding and assembly of VLPs.

Therefore, in terms of insertion capacity, HaPyV-VP1-derived VLPs belong to the group of VLPs with a high insertion capacity like those tolerating more than 100 foreign aa as bluetongue virus NS1 tubules and parvovirus B19-derived VLPs or even more than 200 foreign aa as HBV core and Ty-derived VLPs. This insertion capacity is much larger than that observed for other VLP carriers, e.g. bacteriophage coat protein-derived VLPs.

Immunization with any of the chimeric VP1-derived VLPs containing VR inserts was able to induce a high-titered IgG antibody response against PUUV-VR N protein in BALB/c mice. The observed endpoint titres for PUUV-VR N protein-specific antibodies in mice immunized with chimeric VP1-derived VLPs harboring VR120-1 or VR120-4 inserts (about 1: 200,000) are in the same range as titers observed in BALB/c and C57/Bl6 mice immunized with HBV core particles harboring 120 N-terminal aa of N proteins from PUUV, Dobrava or Hantaan hantaviruses. Although the use of adjuvants induced about 10fold higher titers of PUUV-VR N-specific antibodies in mice immunized with VP1/VR120-1 and VP1-VR120-4, immunization without adjuvants also provided long-term humoral immune response against PUUV-VR N protein and induction of antigen-specific T-cell help.

A major drawback for the application of chimeric VLPs as vaccines may represent a pre-existing immunity directed against the VLP carrier. We observed that the insertion of N protein segments reduced the VP1 carrier antigenicity and immunogenicity. The level of reduction is related to the size of the foreign insertion; i.e. the larger the insert the lower the remaining VP1 antigenicity and immunogenicity. This might represent an additional advantage of chimeric HaPyV-derived VLPs in terms of vaccine development where a low antigenicity and immunogenicity of the carrier itself is wanted.

In addition to used for insertion internal sites, the carboxy-terminal region of HaPyV-VP1 was also predicted to be at least partially surface exposed and demonstrated to represent an immunodominant and highly cross-reactive part of VP1. Due to its putative involvement in the interaction of pentameric subunits of the virion this VP1 region of HaPyV and other polyomaviruses has never been explored as a potential insertion site for foreign sequences. We investigated the potential influence of the carboxy-terminal region of HaPyV-VP1 on the assembly of VLPs using carboxy-terminally truncated or modified VP1 proteins expressed in yeast. The ability of yeast-expressed VP1 variants to form virus-like particles (VLPs) strongly depended on size and

position of truncations. VP1 variants lacking 21, 69 and 79 amino acid (aa) residues at its carboxy-terminal region efficiently formed VLPs similar to those formed by the unmodified VP1 (diameter 40-45 nm). On the contrast, VP1 derivatives with carboxy-terminal truncations of 35 to 56 aa residues failed to form VLPs. VP1 mutants with a single A336G aa exchange or internal deletions of aa 335 to aa 346 and aa 335 to aa 363 resulted in the formation of VLPs of a smaller size (diameter 20nm). These data indicates that certain parts of the carboxy-terminal region of VP1 are not essential for pentamer-pentamer interactions in the capsid, at least in the yeast expression system used, and might be used as additional insertion sites for foreign protein segments and epitopes.

Polyomavirus-derived VLPs were used as tools to study uptake mechanisms of polyomavirus-derived VLPs by human DCs (hDCs) and their maturation. For the uptake of HaPyV-VP1-derived VLPs by human DCs studies a VP1/enhanced green fluorescent protein (eGFP) fusion was generated allowing the investigation of the uptake by its intrinsic fluorescence. Alternatively, the uptake of VP1-derived VLPs of HaPyV and other polyomaviruses was studied by labelling with 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFDA) and subsequent competitive inhibition experiments. Finally, the maturation of hDCs was measured after addition of VP1-derived VLPs originated from different polyomaviruses. Monocyte-derived hDCs were found to internalise VP1-derived VLPs with different levels of efficiency and mechanisms between the VLPs of the tested polyomaviruses. Exposure to VLPs originating from MPyV HaPyV thereby induced maturation of hDCs and CD8-positive specific T-cell responses in vitro, whereas VLPs derived from human polyomaviruses (BK and JC) as well as SV40 were less potent. These data offer important implications for development of polyomavirus VLP-derived vaccines and gene transfer carriers as well as polyomavirus-mediated tumor induction and progression.

Capability of HaPyV-VP1-derived VLPs to tolerate foreign protein segments of up to 120 aa at certain sites of VP1, to induce a strong insert-specific antibody response, even in the absence of any adjuvant and stimulate hDCs was explored for human tumor vaccine development based on HaPyV-derived VLPs presenting tumor antigen CTL epitopes. VP1 was used for the insertion of a human CTL tumor epitope carrying a 9 amino acid (aa)-long cytotoxic T-cell epitope (STAPPVHNV) of human mucin 1 (MUC12A) at different sites of a single VP1 molecule. VP1 VLPs well tolerated two MUC12A peptide inserts. VLPs harboring MUC12A epitopes were able to induce a strong DC maturation evaluated by immunophenotypic DC maturation markers CD83, CD80, CD86, HLA ABC or HLA DR DQ, the secretion of the cytokine IL-12 and decreased capacity of antigen capturing. The co-cultivation of chimeric VLPs loaded DCs with autologous peripheral blood leukocytes resulted in MUC12A epitope specific cytotoxic T cell proliferation and elevated numbers of IFN gamma secreting CD8+ T cells. In addition, specific antibodies against MUC12A epitope were induced in mice only when it was presented on VLPs but not conjugated with BSA. The chimeric VP1 VLPs uptake, processing and maturation by human DCs, induction of MUC12A epitope specific human T cell stimulation in vitro and antibody induction in mice demonstrate the value of chimeric hamster polyoma VP1 VLPs in immunotherapy. This might have implications for the application of this VLP platform in tumour vaccine development.

Very successful was employment of HaPyV-VP1-derived chimeric VLPs as tools for hybridoma technology to generate monoclonal antibodies (mAbs) of desired specificity. Chimeric VLPs containing inserts of different size and origin (MUC12A and PUUV-VR N protein 120 aa segment) were used as immunogens. The immunizations of mice were performed in Laboratory of Immunology and the results of this study are presented there.

In conclusion, HaPyV VP1-derived VLPs are efficiently taken up by human and murine DC, induce DC maturation, represent efficient carriers for foreign peptide insertions and could be successfully employed for vaccine development as well as for hybridoma technology.

Hantavirus vaccine and diagnostic tools

Project was supported EC Framework 5 programme, project QLK2-CT-1999-01119. The project was started in 2000, and finished in 2005.

Hantaviruses represent a separate genus *Hantavirus* of the family Bunyaviridae. They contain a tripartite RNA genome of negative polarity. The genome segments M (medium) and L (large) encode a glycoprotein precursor which is co-translationally processed into G1 and G2, and an RNA-dependent RNA polymerase. The S (small) genome segment codes for the nucleocapsid (N) protein. We have described the high-level yeast expression of authentic and amino-terminally His-tagged rN protein of PUUV (strain Vranica/Hällnäs) which are able to induce a protective immune response in bank voles, the natural host of PUUV (Dargevičiūtė et al., 2002).

High-level expression in yeast and purification of high yields of rN proteins of hantavirus species originating from Asia (HTNV)

and different regions of Europe (PUUV strains Sotkamo and Kazan from Finland and Russia, respectively; DOBV-Slk from Slovakia and DOBV-Slo from Slovenia) was also performed. The rN proteins were characterized in terms of stability, nucleic acid and endotoxin contamination, antigenicity and immunogenicity.

Hantavirus N can be expressed at high level in yeast.

The yields of purified rN proteins from the different hantaviruses mentioned above were reproducibly high ranging from 0.5 to 1.5 mg per g wet weight of yeast cells.

The main portion of rN protein in yeast remains insoluble and forms aggregates.

In line with observations during the expression of hantavirus rN proteins in *E. coli*, the yeast-expressed rN proteins of PUUV-Vra and the other hantaviruses described here were highly insoluble, requiring 8M urea for solubilisation. Nevertheless, as evidenced previously already for PUUV-Vra rN protein (Dargevičiūtė et al., 2002), the rN proteins are highly reactive with mAbs in Western blots. Moreover, rN proteins of PUUV-Vra, HTNV-Foj and DOBV-Slk have been used to establish highly specific and sensitive IgG ELISAs for the detection of antibodies in human sera.

Yeast-expressed rN proteins are highly pure and stable during long-term storage.

In terms of vaccine development such properties as protein stability, nucleic acid contamination and endotoxin contamination are worth to investigate. The yeast-expressed rN proteins are stable in yeast; when analysing yeast lysates obtained after induction of heterologous protein synthesis we did not observe a significant degradation of rN proteins in Western blots.

The nucleic acid contamination of PUUV-Vra rN protein determined by a dot-blot method was found to be very low (about 6.4 pg per 100 µg). This level of DNA contamination is much lower than the contamination accepted for commercial vaccines for human use of 100 pg per dose (see Center for Biologics Evaluation and Research and FDA, 1993).

Yeast-expressed rN proteins induce a strong and cross-reactive antibody response in rabbits.

In line with immunisation data in BALB/c and C57BL/6 mice the yeast-expressed rN proteins induced a high-titered, cross-reactive antibody response in rabbits reaching reciprocal end point titres of up to 10^5 to 10^6 for the corresponding homologous antigen.

Hantavirus seroprevalence was investigated in Lithuania.

Recently we started to investigate Hantavirus distribution in Lithuanian population (Sandmann et al., 2005). Two serum panels from cancer patients (n = 438) and blood donors (n = 299) from Lithuania were tested by monoclonal antibody capture IgG ELISA using yeast-expressed recombinant nucleocapsid (rN) proteins of Puumala virus (PUUV), Hantaan virus (HTNV) and Dobrava virus (DOBV). Selected serum samples were further analyzed by focus reduction neutralization assays. In our study we found a wide distribution of hantavirus-positive samples in Lithuania; in all seven counties investigated hantavirus-reactive samples were identified.

At least two different Hantaviruses (PUUV and DOBV) have been detected in Lithuania. DOBV dominance is noted (fig 1). Additional investigations are pursued to study the seroprevalence more precisely and to search for clinical cases of hantavirus infections.

In conclusion, our yeast expression system is very efficient for the generation of rN proteins of different hantaviruses origina-

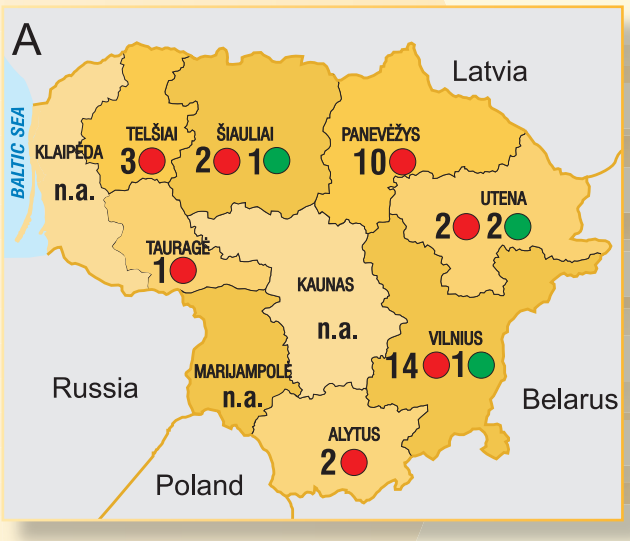

Figure 1. Map of Lithuania with the localization of the origin of the 36 ELISA-reactive, immunoblot-confirmed human serum samples in the different counties of Lithuania (Sandmann et al., 2005).
Red circles: Hantaan Dobrava, Hantaan Dobrava>Puumala, Hantaan and Dobrava-reactive in the IgG ELISA;
Green circles, Puumala and Hantaan Dobrava<Puumala-reactive sera;
n.a., not analyzed

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ted from Asia and Europe. An amino-terminal His-tagging enables an easy purification of hantavirus rN proteins. The His-tagged rN proteins were found to be highly stable during long term storage and to contain only very small traces of nucleic acid contamination. Moreover, immunisations of rabbits confirmed the high immunogenicity of the yeast-expressed rN proteins of the different hantaviruses. Therefore, non-infectious, yeast-expressed hantavirus rN proteins represent promising vaccine candidates. In addition, these rN proteins are helpful tools for establishing specific and reliable assays for hantavirus diagnostics.

Enhanced surveillance of measles

Project was supported EC Framework 5 programme, project QLK2-CT-2001-01632. The project was started at 2002.

Measles virus, belonging to the *Morbillivirus* genus of a family *Paramyxoviridea*, is an enveloped virus, which encapsidates a 16-kb negative strand RNA genome (for review, Griffin, 2001). Mature particles are assembled from six viral-encoded proteins. Two glycoproteins, the hemagglutinin and the fusion protein, are the major protein constituents of the envelope. A third protein, a matrix protein, lines the interior surface of the viral envelope and is believed to link glycoproteins to the internal viral proteins. The nucleoprotein (MeN), the major internal protein, has appears to be responsible for viral genome packaging and formation of replication complexes along with other proteins. Two additional proteins, namely, the phosphoprotein and large protein or RNA polymerase, are associated with nucleocapsid and are involved both in replication and transcription of viral RNA. Viral nucleocapsid proteins usually elicit a strong long-term humoral immune response in patients and experimental animals. Therefore, measles diagnosis can be based upon the detection of antibodies specific to MeN. The main task of the Institute of Biotechnology, laboratory of Eukaryote genetic engineering was the development of a simple, efficient and cost-effective system for generation of measles virus nucleoprotein for measles serology.

The gene encoding measles virus Schwarz strain nucleoprotein was successfully expressed in two different yeast genera, *Pichia pastoris* and *Saccharomyces cerevisiae*, respectively. Both yeast genera synthesized a high level of nucleoprotein, up to 29% and 18% of total cell protein, in *Pichia pastoris* and *Saccharomyces cerevisiae*, respectively. This protein is one of most abundantly expressed in yeast. Convalescent sera from measles patients reacted with the recombinant protein and a simple diagnostic assay could be designed on this basis. After purification nucleocapsid-like particles derived from both yeast genera appeared to be similar to those detected in mammalian cells infected with measles virus. A spontaneous assembly of nucleoprotein into nucleocapsid-like particles in the absence of the viral leader RNA or viral proteins has been shown. Compartmentalization of recombinant protein into large compact inclusions in the cytoplasm of yeast *Saccharomyces cerevisiae* by GFP fusion has been demonstrated. Monoclonal antibodies against recombinant measles nucleocapsid protein were generated in the Laboratory of Immunology and Cell Biology. Characterization of these antibodies described in the section of The Laboratory of Immunology and Cell Biology. The purified rMeNP and a monoclonal antibody to the rMeNP conjugated to horseradish peroxidase were used to develop a measles specific IgM capture EIA, MACEIA, in both serum and oral fluid specimens. Evaluations of the MACEIA were performed by testing a) serum samples (n=80), b) paired oral fluid / serum samples (n=37, representing 15 cases) and c) oral fluid samples from cases of

measles notified by general practicioners (n=145). The samples were also tested by measles IgM radioimmunoassay, MACRIA. The sensitivity and specificity of the MACEIA compared to MACRIA for a) the serum samples were 100% and 96.6% respectively, b) for paired serum / oral fluids samples 100% and 100% respectively and c) for oral fluid samples 96.6% and 100%

Conclusion: Recombinant yeast derived measles nucleocapsid protein efficiently formed nucleocapsid – like particles in both yeast genera, *S.cerevisiae* and *P.pastoris*. Yeast generated MeNP nucleocapsid-like particles are suitable for the development of very sensitive measles specific IgM and IgG detection kits.

West Nile virus

The project was partially supported by EC FR6 programme project LSHB-CT-2004-005246 "Rational design and standardized evaluation of novel genetic vaccines". Project was started at 2005.

West Nile virus (WNV) is an emerging, mosquito-borne human pathogen that first appeared in USA in 1999 from Africa and currently constitutes a serious health treat. Sporadic outbreaks have also occurred in Europe, and the virus has been isolated from birds in UK. WNV is a member of the *Flaviviridea* family, which includes many important human pathogens, like dengue, yellow fever, tick-borne encephalitis, and hepatitis C viruses.

The task of our team in this project was generation of WNV proteins in yeast and generation of selected WNV epitopes as virus-like particles on the basis of MuPyV VP1 VLPs.

The expression of WNV core protein in yeast was unsuccessful, no detectable additional band in SDS-PAGE was observed. One of reasons of such results could be comparatively high level of predicted yeast splicing signals in this gene. Expression of surface protein E in yeast was rather successful. This protein was purified as VLPs by ultracentrifugation in CsCl gradients and as a amorphous protein by using Ni-chelate techniques. WNV E protein purified by ultracentrifugation forms small particle-like structures.

The main antigenic loop of WNV E protein was inserted into HI loop of MuPyV VP1. Electron microscopy of purified protein confirmed, that such chimeric protein formed virus-like particles. All WNV recombinant proteins were delivered to the project partner Erasmus Medical Centre, Rotterdam for evaluation of immunogenicity. WNV E protein will be used for study of WNV antibodies in sera of Lithuanians birds also.

Seroepidemiology of the human polyomaviruses

Project was carried out in the cooperation with Dept. of Medical Microbiology, Malmö University Hospital and HPA, Colindale, London.

Human polyomaviruses are endemic and infect a large proportion of mankind all over the world. Primary infections with the human polyomaviruses BK and JC virus occur during childhood and are largely asymptomatic. Following primary infection, both BKV and JCV persist as latent infections in the kidneys and B-lymphocytes.

Under conditions of severe immunosuppression such as leukaemia, AIDS and organ transplantation both viruses may be reactivated and cause diseases. Reactivation of BKV is mainly related to urinary tract diseases like haemorrhagic cystitis, ureteric stenosis, glomerulonephritis, and graft nephropathy . The JC virus can be reactivated and induce the CNS disease progressive multifocal leukoencephalopathy, PML.human polyomaviruses have in vitro transforming abilities, similar to the mouse polyomavirus and the monkey polyomavirus SV40. In recent years a possible association of polyomaviruses with human cancer has been reported. The JC virus has been detected in certain brain tumors, in particular oligoastrocytoma. The monkey polyomavirus SV40 was present in polio vaccines produced in rhesus monkey kidney cells during the 1950s and SV40 has been detected in several human tumors, including choroid plexus tumors and ependymomas. The BK virus has also been detected in a variety of tumors, including neuroblastoma.. These reports have created a renewed interest in the epidemiology of these infections.

Hemagglutination inhibition test (HI) has been the standard method for measurement of antibody titres to BK virus and JC virus. About 70-90 % of healthy adults are seropositive by HI. Seroconversion for BK infection occurs in early childhood and JC seroconversion occurs in late childhood. A study from the United States reported antibodies to BKV among 50 % of children in the ages 3-4 years and that almost all children were seropositive at the age of 10 years. Antibodies to JCV were acquired by 50 % of children aged 10-14 years and about 75 % of adults.

In recent years viral serology based on virus like particles in enzyme immunoassays (EIA) has become widely used. EIA's using specific antigens of BKV and JCV has shown a greater sensitivity and precision compared to HI.

To assess stability over time of polyomavirus antibodies in serial samples and the incidence and age specific prevalence of these infections, we established enzyme immunoassays using purified yeast-expressed virus like particles (VLP) containing the VP1 major capsid proteins of JC virus and BK virus strains AS and SB, respectively. A random subsample of 150 Finnish women who had serum samples taken during their first trimester of pregnancy and had two consecutive pregnancies during a 5-year follow up period was selected and stratified by the age at first pregnancy. The polyomavirus antibody levels were similar in samples taken at the first and second pregnancy (Correlation coefficient: 0.93 for BKV SB and 0.94 for JCV). Analysis of serum samples from 290 Swedish children in the ages 1-13 years, stratified by age in 2 year-intervals, demonstrated that BKV seropositivity increased rapidly with increasing age of the children, reaching 98% seroprevalence at 7-9 years of age, followed by a minor decrease. JCV seroprevalences increased only slowly with increasing age, reaching 72% positivity among mothers >25 years of age.

In conclusion, the age-specific seroprevalences of the human polyomaviruses, as measured using a VLP-based EIA, were similar to previous serosurveys by other methods. The stability over time indicates that polyomavirus seropositivity is a valid marker of cumulative virus exposure and polyoma VLP-based EIAs may therefore be useful for epidemiological studies of these viruses.

The objectives of the second study were to establish a modern seroepidemiological technology based on yeast expressed virus like particles of JC virus, the two major BKV strains AS and SB and SV 40, to investigate stability over time of polyomavirus antibodies in serial samples and to investigate the incidences and age specific prevalences of these virus infections.

The human polyomaviruses have in vitro transforming abilities and JCV DNA has been reported to be present in human colon mucosa as well as in colorectal cancers.

To investigate whether the risk for colon cancer is associated with JCV or BKV infection, we performed a case-control study nested in a cohort of 600,000 healthy Norwegian subjects that was followed prospectively for 30 years using registry linkages. Three-hundred seventy-eight cases of colorectal cancer with baseline serum samples taken >3 months before diagnosis were identified and matched for sex, age, date of blood sampling and county of residence with 378 control subjects who had not developed colorectal cancer at the time of diagnosis of the matched case. JCV and BKV exposure status was determined using detection of specific IgG to JCV or BKV virus like particles. Validation of the serum assay found high (97-100%) sensitivity for detection of subjects with viral DNA detectable in urine and ability to discriminate between shedders of the different polyomaviruses. Analysis of a subset of subjects with serial samples taken a year apart found the antibodies to be mostly stable over time.

The relative risk for colorectal cancer among JCV seropositive subjects was 0.9 (95% CI: 0.7 - 1.3) and the BKV-associated relative risk was 1.1 (95% CI: 0.8 - 1.5). Calculation of relative risks at different cut-offs for determining seropositivity also found no evidence of excess risk.

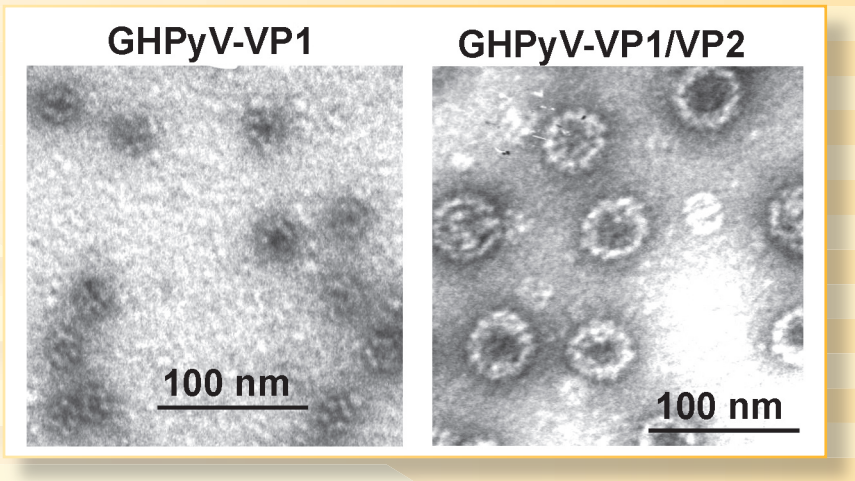
In summary, a prospective study using a serologic assay validated to measure JCV and BKV infections found no evidence for association between these infections and excess risk for colorectal cancer.

Generation of Goose Hemorrhagic Polyomavirus Virus-Like Particles and their Application in Serological Tests

Goose hemorrhagic polyomavirus (GHPV) has been identified as the causative agent of hemorrhagic nephritis and enteritis of geese (HNEG), a devastating disease with high mortality rates in young geese The disease has been first described in 1969 in Hungary and appeared subsequently in Germany and France Recent outbreaks of HNEG with mortality rates up to 44 % and 67 % have been described in Germany and Hungary, respectively. HNEG is characterized by nephritis, enteritis, ascites, and oedema of the subcutaneous tissues in 2-10-week-old geese.

GHPV exhibits the typical characteristics of a polyomavirus. Until now, GHPV cannot be efficiently propagated in tissue culture rendering antigen production for use in diagnostic tests and vaccines difficult.

To provide antigens for diagnostic tests and vaccines, its major structural protein VP1 was recombinantly expressed in *Sf9* insect cells and in the yeast *Saccharomyces cerevisiae*. As demonstrated by density gradient centrifugation and electron microscopy, GHPV-VP1 expressed in insect cells formed virus-like particles (VLPs) with a diameter of 45 nm indistinguishable from polyomavirus particles. However, efficiency of VLP formation was low as compared to the monkey polyomavirus SV-40-VP1. In yeast cells, GHPV-VP1 alone formed smaller VLPs, 20 nm in diameter. Remarkably, co-expression of GHPV-VP2 resulted in VLPs with a diameter of 45 nm. All types of GHPV-VLPs were shown to hemagglutinate chicken erythrocytes. An ELISA and a hemagglutination inhibition (HI) assay established on the basis of the VLPs revealed a high seroprevalence in flocks of geese with HNEG and no antibodies in a clinically healthy flock. However, GHPV-specific antibodies were also detected in some other



VLP formation of purified GHPV-VP1 and GHPyV-VP1/VP2 VLPs expressed in yeast determined by negative staining electron microscopy

flocks without HNEG indicating a broad distribution of the GHPV infection due to subclinical or unrecognised infections. Application of the established ELISA and the HI assay could contribute to the understanding of the epidemiology of the GHPV infection and help to assess the distribution and spreading of the virus within the geese population.

Enhanced surveillance of respiratory viruses

The project was supported by Lithuanian Science and Study Foundation, Project B-11/2003-2006. Enhanced surveillance of respiratory viruses.

Respiratory tract viruses of *Paramyxoviridea* family like viruses hPIV 1, 2, 3 and hRSV are the major cause of croup as well as pneumonia, bronchiolitis and other serious respiratory tract diseases in infant, children and older adults. The recent outbreak of SARS confirms the importance of surveillance of Coronavirus.

Development of the fast and convenient methods for detection and classification of most important respiratory virus would complement clinical diagnosis and speed up curing of the disease. The objective of the project is developing of fast and convenient enzyme-immunoassays based methods for key respiratory viruses. Development of the fast and convenient methods for detection and classification of most important respiratory virus would complement clinical diagnosis and speed up curing of the disease. The project is directed towards developing of fast and convenient enzyme-immunoassays based methods for the key respiratory viruses. In 2003 nucleocapsid (NP) genes of hPIV1, RSV-Sendai, hRSV-A and SARS virus were modified for cloning into yeast expression vectors and nucleotide sequences were determined. In 2004 producers of hRSV- A, hRSV-B, hPIV1, hPIV3, Rubella and SARS viruses of nucleocapsid proteins, and SARS virus SP, were constructed. An additionally, the rubella virus proteins were included also in the cooperation with HPA, London. The corresponding proteins were purified and characterized. It was demonstrated that Sendai, hRSV-A, hRSV-B, hPIV1 and hPIV3 virus nucleocapsid proteins efficiently formed virus-like particles (VLPs) in yeast. In 2005 the construction of hybridomas against hPIV1, hPIV3 and Rubella nucleocapsid proteins were finished and corresponding IgG monoclonal antibodies prepared. Clinical samples were collected and characterized using commercial test kits. Recombinant antigens and monoclonal antibodies were used to develop IgG/IgM serologic assays that were evaluated by testing characterized clinical samples. Serologic assays for Rubella-specific antibodies were evaluated in collaboration with project partners from HPA (London). The project is carried out in cooperation with the laboratory of Immunology and Cell Biology.

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Laboratory of Biothermodynamics and Drug Design

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Establishment of the Laboratory of Biothermodynamics and Drug Design (LBDD)

The laboratory was established only recently – January 1, 2006 – a year after its head, Dr. Daumantas Matulis returned from United States where he obtained Ph.D. from University of Minnesota and worked for Johnson&Johnson Pharmaceutical Research and Develompent, L.L.C. The new laboratory is based on the Laboratory of Recombinant Protein Research and retained some of the experienced personnel in this field.

Organizational Structure of LBDD

The laboratory is highly interdisciplinary and consists of four closely interacting groups of biomedical scientists:

1. Recombinant protein production
2. Organic synthesis
3. Biothermodynamics of protein-ligand interaction
4. Computer simulation of protein-ligand interaction

The group of recombinant protein production clones genes of selected target proteins, expresses them in *E.coli*, insect, or mammalian cells, and purifies large quantities of active proteins sufficient for biothermodynamic measurements of binding with synthesized ligands. Several projects involve design of protein domain constructs with targeted mutations in order to demonstrate various amino acid contributions to the energetics of ligand binding. Protein production often involves reconstitution and refolding of proteins from insoluble inclusion bodies.

The group of organic synthesis makes compounds that are anticipated to bind target proteins either by comparison with compounds of similar chemical structure or by computer simulation. Special interest and capabilities of the group are in the field of synthesis of compounds with multiple conjugated aromatic heterocycles.

The group of biothermodynamics of protein-ligand interaction is making measurements by two biophysical techniques, namely, isothermal titration calorimetry (ITC) and protein melting temperature shift (PMTS). The ITC capability has been recently added by the purchase of Nano ITC titration calorimeter from Setaram (France), made by Calorimetry Sciences Corporation (USA). In addition, the binding constants are confirmed by PMTS using fluorimeter with temperature control. Furthermore, the group is involved in the studies of protein denaturation by high pressure using ISS high pressure fluorimeter that has been recently donated to our laboratory by Johnson&Johnson PRD, L.L.C. (USA).

The group of computer simulation of protein-ligand interaction consists of several collaborating scientists that reside outside Lithuania. They are developing software that estimates the energetics of ligand binding to a protein when only the crystal structure of unbound protein and chemical structure of the potential ligand is available.

Lina Mištinaitė, B. Sc.

Jurgita Matulienė, Ph. D.

Research Projects

Since the LBDD has been established so recently, there are no significant results, publications or presentations based on the work performed here. The laboratory selected several protein targets and anticipated several potential ligands of interest to be synthesized.

In the attempt to inhibit signal transduction in the proliferation of cancerous cells, the protein-protein interaction of Hdm2 with p53 is probed with compounds mimicking three hydrophobic amino acids exposed on the surface of p53 that bind to three complementary pockets on the surface of Hdm2.

Second project involves discovering sulfonamides that would specifically inhibit only one or several carbonic anhydrases out of about 15 known carbonic anhydrase isoenzymes. Several of them are known to be involved in the development of various cancers. The project continues building on our previous results.

The former laboratory of recombinant protein research has significant experience in purification and folding of chaperone proteins. One of them, heat shock protein 90 (Hsp90), is an anticancer target. The search and synthesis of compounds similar to well known inhibitors of Hsp90, namely, radicicol and geldanamycin, are underway.

Collaborations

Cathy Royer, Montpellier, France – high pressure fluorimetry

Matthew J. Todd, Barry Springer, Johnson&Johnson, PRD, L.L.C., Philadelphia, USA – high throughput PMTS (ThermoFluor®) method

Grants

EC FP6 Marie Curie international reintegration (Daumantas Matulis)

Lithuanian State Science and Studies Foundation (Daumantas Matulis)

EC FP6 Marie Curie international reintegration (Jurgita Matulienė)

Sebastien Durand from Setaram, France.

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Liodzia Diglienė, technician

The Laboratory of Immunology and Cell Biology (former name - Laboratory of Immunology) consists of several research groups. During 2004-2005, the research was focussed to the following topics: immunogenicity studies of recombinant viral proteins (Dr. A. Zvirbliene), regulation of gene expression by alternative splicing (Dr. A. Kanopka), regulatory role of molecular chaperones in the cell signalling processes (Dr. A. Kazlauskas), molecular epidemiology of tuberculosis (Dr. P. Stakenas), studies on the epigenetic variability in germ cells (Dr. V. Popendikyte). Since 2006, the epigenetic studies will not be continued as the group leader Dr. V. Popendikyte has left the institute.

During 2004-2005, the Laboratory has organized two workshops supported by the Biocel project: "Molecular diagnostics of respiratory diseases" (March 2005, Dr. P.Stakenas), „The markering of human, animal and microorganism genomes by using PCR-based technologies" (Oct 2005, Dr. V.Popendikyte).

Immunogenicity studies of recombinant viral proteins

This work was performed in collaboration with the Laboratory of Eukaryote Gene Engineering. It was aimed at evaluating the antigenic and immunogenic properties of yeast-expressed recombinant proteins that might be exploited as potential vaccines or diagnostic tools.

1. Characterization of the antigenic structure of measles nucleoprotein.

The B-cell response against measles nucleoprotein (MeN) plays an important role in the control of measles infection. However, the data on B-cell epitopes of MeN are still limited. The objective of this study was to identify B-cell epitopes in MeN using monoclonal and polyclonal antibodies raised against recombinant yeast-expressed MeN as well as human sera from infected and vaccinated individuals.

Recent structural studies of MeN revealed that it consists of two regions: the N-terminal (N_{CORE}) with conserved sequences required for self-assembly and RNA binding; and a C-terminal (N_{TAIL}) which is intrinsically disordered and protrudes from the viral nucleocapsid surface. Localization of B-cell epitopes may provide additional information on the structure of MeN since the antigenic parts of a protein's surface are predominantly located in the loops and/or protruding regions.

In order to locate the the precise antibody-binding regions on the MeN, we have employed recombinant overlapping GST-fused MeN fragments and a set of overlapping synthetic peptides (*PepScan* analysis). Using this approach, we have identified several immunodominant regions within the C-terminal region of the rMeN. Most of monoclonal antibody epitopes were mapped between 419 and 525 aminoacids. *PepScan* analysis and cross-inhibition studies with human sera demonstrated essentially the same localization of B cell epitopes recognized by serum antibodies from vaccinated and naturally infected individuals. In conclusion, these findings demonstrate an accesibility of C-terminal domain of MeN to B cells and are in agreement with recent experimental evidences indicating that the N_{TAIL} is well-exposed on the surface of nucleocapsid-like structures.

2. Investigation of the immunogenicity of chimeric virus-like particles.

Recombinant virus-like particles (VLPs) harbouring foreign epitopes at certain surface-exposed positions are highly immunogenic and represent a promising universal carrier for various vaccine development applications. Because of their repetitive antigenic structure, chimeric VLPs can induce a strong insert-specific humoral immune response. Recently, we have demonstrated that yeast-expressed hamster polyomavirus major capsid protein VP1-derived VLPs well tolerated the insertion of foreign sequences of different size and origin at four predicted surface-exposed sites. The insertion into VP1 did not influence the assembly of VLPs. Immunisation of mice with these chimeric VLPs resulted in the induction of a high-titered insert-specific antibody response (*Gedvilaite et al., 2004*). The current study was aimed at evaluating VP1-derived VLPs as carriers for hybridoma technology to generate monoclonal antibodies (mAbs) of desired specificity. For this purpose, chimeric VLPs harbouring 9 and 120 aminoacid (aa)-long foreign sequences were generated. Chimeric VLPs harbouring a CTL epitope of human mucin 1 at previously defined insert positions #1 and #4 elicited in mice a strong humoral immune response against the inserted epitope and promoted the production of epitope-specific mAbs. From 7 mAbs generated against the chimeric VLPs, 2 mAbs were specific to the inserted epitope and 5 mAbs to the VP1-carrier. As the number of antigen-positive hybridoma clones correlates with the yield of antigen-positive B cells in the spleen, this shows that the inserted epitope elicited an efficient B cell response in spite of its small size as compared with the VP1-carrier (9 aa and about 380 aa, respectively). Moreover, all hybridomas were of IgG isotype, which indicates that the VLPs induced an efficient T cell help required for immunoglobulin isotype switch. The predominant activation of insert-specific B cells was more obvious with the chimeric VLPs harbouring a single 120 aa-long segment of hantavirus nucleocapsid protein. The immunogen promoted the induction of mAbs of IgG isotype directed exclusively to the inserted hantavirus nucleocapsid protein sequence. The mAbs recognized full-length yeast-expressed hantavirus

nucleocapsid protein and native nucleocapsid protein in hantavirus-infected mammalian cells (Fig. 1). This suggests a correct folding of the nucleoprotein sequence displayed on VLPs.

The generation of mAbs against the VP1-carrier permitted detailed characterization of its antigenic structure. The epitopes of 2 mAbs were mapped at the previously defined insert site #2 within the EF loop (aa 223/224), which confirms its surface-exposed localization. One epitope was localized at the amino-terminus of VP1 between 59 and 64 aa, corresponding to the beginning of BC loop. This suggests a new potential insert site. Thus, the use of mAbs provided new data on the immunodominant, B cell accessible regions of the VP-carrier, that correspond well with earlier prediction and experimental data regarding the surface-exposed sites of hamster polyomavirus VP1.

As evidenced for different VLPs, their strong immunogenicity is driven by activation of dendritic cells (DCs). Therefore, we have evaluated the ability of hamster polyomavirus VP1-derived chimeric VLPs to activate antigen-presenting cells by studying the uptake of these chimeric VLPs by murine spleen cell-derived DCs. An efficient uptake of VP1-derived fluorescent VLPs by murine DCs was demonstrated (Fig. 2). In addition, the chimeric VLPs were capable to induce cytokine release in DC cultures and DC/T helper cell co-cultures indicative for DC activation and priming of naïve T helper cells. Thus, the efficient uptake of VP1-derived VLPs by DCs and their activation may represent the basis of the strong immunogenicity of these chimeric VLPs.

In conclusion, the present study proves the potential of chimeric VLPs as a new type of immunogens for the production of mAbs with the desired epitope-specificity. The main advantage of this approach over the traditional use of synthetic peptides coupled to carrier proteins is the possibility of predicting the surface localization of a particular sequence. The insertion of non-immunogenic epitopes into VLPs may significantly increase their ability to induce a strong B cell response, which provides an efficient way of producing epitope-directed mAbs. Based on these investigations, the patent application was filed (1013 IP, 2005 043, LT).

This work was supported by the Lithuanian State Science and Studies Foundation (projects T 45/04, T-78/05, B-16/2005).

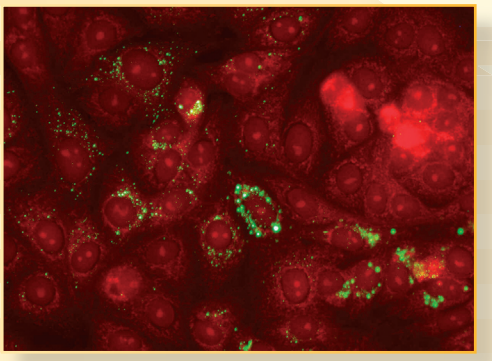

Figure 1. Monoclonal antibodies raised against a segment of hantavirus nucleocapsid protein inserted into VPLs recognize native nucleocapsids in hantavirus-infected cells.

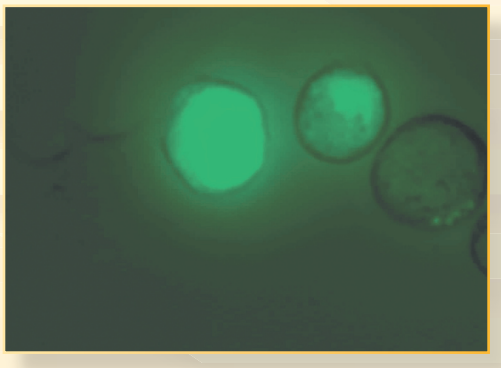

Figure 2. Demonstration of the uptake of fluorescent chimeric VLPs by murine dendritic cells.

Regulation of hypoxia-inducible factor (HIF) expression via the alternative pre-mRNA splicing mechanism.

In eukaryotes, the protein coding sequences of most mRNA precursors (pre-mRNAs) are interrupted by non-coding sequences called introns. Pre-mRNA splicing is the process by which introns are removed and the protein coding elements assembled into mature mRNAs. Alternative pre-mRNA splicing selectively joins different exon elements to form mRNAs that encode proteins with distinct functions, and is therefore an important source of protein diversity.

The pre-mRNA splicing reaction occurs via the two-step mechanism. In the first step, the 5′ end of the intron is joined to the adenine residue located in the branchpoint sequence, upstream the 3′ splice site, resulting in a branched intermediate called

an intron lariat. In the second step, the exons are ligated and the intron is released as lariat. Cryptic splice sites are normally avoided by the splicing machinery, however, they can be selected for splicing when normal splice sites are altered by mutation. Identification of the correct splice sites is achieved by virtue of their proximity to exons. Abnormalities in the splicing process can lead to various disease states. Some of these defects are caused by mutations in the sequences of the gene required for intron recognition and, therefore, result in abnormal processing of the pre-mRNA.

Our research is focussed on the molecular mechanisms that underlie the alternative splicing processes of the hypoxia-inducible factor (HIF) pre-mRNAs, as well as the association of pre-mRNA splicing defects with human deseases in general.

It has recently been discovered that a dominant negative regulator of hypoxia-inducible gene expression (IPAS) is generated in hypoxic cells by alternative splicing from HIF-3α pre-mRNA. In our studies, we aim to understand the molecular mechanisms, which underly the alternative HIF-3α/IPAS pre-mRNA splicing process.

We report that: 1) in the HIF-3α pre-mRNA construct, spanning exon 2 and two 3′ alternatively spliced sites of the exon 3, the proximal 3′ splice site was used resulting in the HIF-3α mRNA product; 2) transcripts spanning the 5′ splice site of the exon 3 and the 3′ splice site of the exon 5 in HeLa nuclear extracts, prepared under normoxic conditions, were spliced (resulting in HIF-3α mRNA) more efficiently compared to the construct encompassing the 5′ splice site of the exon 3 and 3′ splice site of the exon 4 (resulting IPAS mRNA). UV crosslinking results revealed an uncharacterized 50 kDa protein, that interacts with short intron 2 3′ splice site sequences of HIF-3α and IPAS with different efficiency in normoxic HeLa nuclear extracts, suggesting the involvement of this protein in regulation of the alternative HIF-3α/IPAS pre-mRNA splicing process.

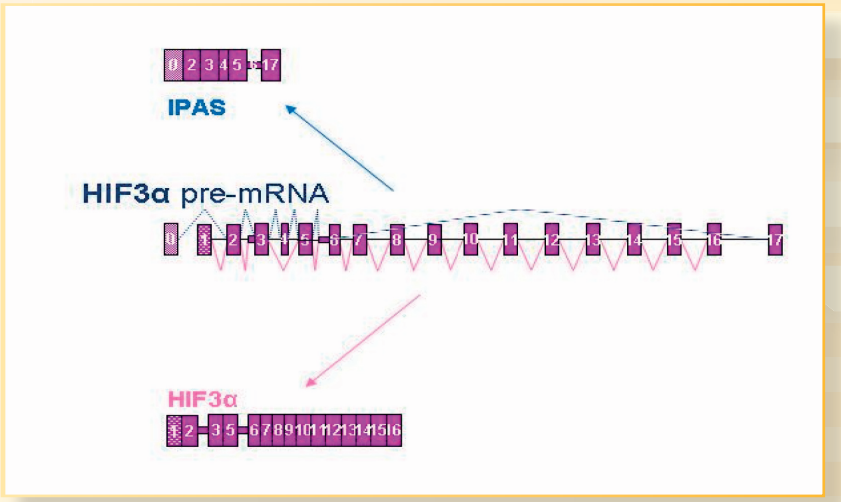

Figure. Schematic illustration of hypoxia-inducible factor HIF-3α pre-mRNA splicing.

This work was supported by EU Framework 6 Programme (project *Euroxy*).

Regulation of bHLH/PAS transcription factor function by the Hsp90 and Hsp70 chaperone complexes

Many cellular proteins require the assistance of molecular chaperones for folding to their native structure [1, 2]. Chaperones are found in all species, from bacteria to higher eukaryotes. Many of them are heat shock proteins (Hsp:s). Hsp70 is involved in folding of nascent polypeptide chains, translocation across the membrane of different cellular compartments (e.g. endoplasmic reticulum or mitochondria) or folding of a broad spectrum of the polypeptides which undergo a conformational damage under cellular stress conditions such as heat shock [2-4]. Hsp90 chaperones do not act generally in nascent protein folding. Instead, most of their known substrates are mediators of cellular signaling, the examples being steroid hormone receptors, protein kinases, such as pp60/v-src and Raf1, and bHLH/PAS proteins such as the the aryl hydrocarbon/dioxin receptor (AhR/DR) and hypoxia-inducible factor-1α (HIF-1α) [4]. A common feature of these substrates is that they undergo initial folding by Hsp70 and then are transferred to Hsp90, the role of which is to stabilize the activation-competent conformation of the client protein [2].

If chaperones fail to generate the native conformation of their target substrate, the unfolded protein eventually meets its fate in the protein degradation pathway, which, in eukaryotic cells, is carried out by the ubiquitin/proteasome system. This system consists of the cascade of ubiquitylating enzymes, the role of which is to mark the target substrate for degradation by covalent

attachment of multiple ubiquitin molecules, and the 26S proteasome complex, which specifically recognizes and degrades ubiquitylated proteins [5].

The main objective of our studies is to elucidate molecular mechanisms that underlie the Hsp90/Hsp70-mediated molecular switch that determines the conformational stability and the turnover rate of the chaperone-dependent cellular signalling molecules. We are currently using two bHLH/PAS transcription factors, AhR and HIF-1α, as model substartes in our experiments. These two proteins represent cellular signalling cascades, that are induced by distinct stimuli: AhR is activated upon ligand binding, whereas HIF-1α is activated by hypoxia. Shortly after being activated, both proteins undergo a modification by plyubiquitylation and subsequent degradation by the 26S proteasome [4]. They are also degraded when their interaction with Hsp90 chaperone complex is compromized. Currently, by using biochemical and cell biology assays, we are examining Hsp70/Hsp90-associated protein factors, such as CHIP and Bag1 [6], that play a role in conveying the polyubiquitylated bHLH/PAS transcription factors to the cellular protein degradation machinery.

This work was supported by the Lithuanian State Science and Studies Foundation (project T-49/05) and EU Framework 6 Programme (project *Euroxy*).

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Molecular epidemiology of *Mycobacterium tuberculosis* in Lithuania

This work was performed in collaboration with Republican Hospital of Tuberculosis and Infectious diseases (Vilnius, Lithuania).

Tuberculosis (TB) is a serious global health problem. An estimated one third of the world's population is infected with *M. tuberculosis* complex bacteria causing nine million of new cases and two million deaths annually. The most effectively tuberculosis is cured according to WHO recommended directly observed treatment short-course (DOTS) strategy that includes treatment regimes using three or four essential antituberculosis drugs. However, an improper management of tuberculosis has led to emergence of multidrug-resistance (MDR), defined as resistance to the two most powerful anti-tubercular drugs isoniazid and rifampin at least. According to DOTS-plus strategy, multidrug-resistant tuberculosis is treated by using the second-line antituberculosis drugs and in the developed countries between 60 and 80% of patients are cured. However, such treatment is far more expensive and in developing nations the majority of patients suffering from multidrug-resistant tuberculosis are condemned to die. Moreover, the average patient may infect a further 15 to 20 people. Therefore, it is of great importance to improve the understanding of the transmission of tuberculosis and the mechanisms of acquisition of drug resistance of *M. tuberculosis*.

In Lithuania, tuberculosis situation remains alarming. There were 2029 notified new and 269 previously treated tuberculosis cases in 2005 (notification rate 66.5 per 100 000 of population). Re-emergence of multidrug-resistant tuberculosis is one of the highest in the world. In year 2002, 9.4% of *M. tuberculosis* isolates recovered from the new and 53.3% isolates from treated previously tuberculosis cases were MDR. In this context, there are strong reasons for increased efforts, including scientific research efforts, to counteract the threatening situation. Therefore, the research group from the Laboratory of Immunology have continued research on molecular epidemiology of *Mycobacterium tuberculosis*. The research is carrying out in collaboration with Republican Tuberculosis and Infectious Diseases University Hospital. The main goal of the past two-year study was to start integration of molecular epidemiology research into National strategy for control of drug-resistant-tuberculosis with the aims to prognosticate possible changes in the population of *M. tuberculosis* and to evaluate the efficiency of implementation of the Strategy as well.

To realize this purpose the following tasks for were set. 1) To characterize population of *M. tuberculosis* strains circulating in Lithuania before the start of treatment of multidrug-resistant tuberculosis according to the DOTS-plus pilot project. 2) To

evaluate the frequency of reinfection with *M. tuberculosis*. 3) To evaluate epidemiological situation in closed communities with increased risk for infection, such as the prisons. 4) To search for mutations that confers *M. tuberculosis* resistance to streptomycin and to the reserve drugs used for treatment of multidrug-resistant tuberculosis with the aim to select the methods for fast resistance prediction.

In total, 223 *M. tuberculosis* isolates including 105 MDR isolates recovered from different patients suffering from tuberculosis were investigated by internationally standardized restriction fragment length polymorphism (RFLP) typing using the insertion element IS*6110* as a probe. Analysis of drug-susceptible genotypes revealed that 13% of isolates fell into small clusters. These data indicated that no of large outbreaks from the common sources have occurred among the patients suffering from drug-susceptible tuberculosis during the year 2005. However, 36% of drug-susceptible isolates belonged to four large groups of highly virulent and genetically related strains identified previously indicating on endogenous origin of these families. In contrary, among multidrug-resistant isolates 57% fell into clusters with identical DNA fingerprints. Moreover, all clustered isolates from new tuberculosis cases were in mixed clusters with isolates recovered from previously treated patients. Ninety-one percents of MDR isolates and 83% of clustered isolates belonged to the large groups of highly virulent genetically related strains mentioned above. In sum, the research indicated that a limited number of especially virulent strains are responsible for the majority of MDR TB cases. Therefore, one of essential things for control tuberculosis situation in Lithuania is to break the transmission chains of these strains. It was determined that among 17 patients suffered from tuberculosis in Lukiskes prison in the year 2005 nobody had epidemiological links. To evaluate whether chronic and secondary cases of tuberculosis could be linked with exogenous reinfection with *M. tuberculosis* the secondary episodes of the disease of 41 patients during three years study were investigated. Reinfection with different *M. tuberculosis* strain was confirmed for six (14.6%) cases. For the remaining patients the secondary episodes of tuberculosis resulted from the failure of treatment.

A prevalence of the mutations in the *rrs* gene associated with *M. tuberculosis* resistance to streptomycin was investigated by developed Polymerase Chain Reaction (PCR)-based assay and direct sequencing. Out of 194 streptomycin-resistant isolates that lacked mutations in the *rpsL* gene, 29.5% isolates carried mutations in the *rrs* gene. Thus, the research on detail characterization spectrum of the mutations among the largest collection of 468 streptomycin-resistant isolates has been finished. The results of this study indicate that genotypic susceptibility for streptomycin tests must include a fixation of the mutations in the codons 43 and 88 of the *rpsL* gene and the mutational changes in the 520-loop of the *rrs* gene.

A simple PCR-based technique for the rapid detection of the mutations in the 5′ region of the *rrs* gene associated with *M. tuberculosis* resistance to kanamycin, amikacin, and capreomycin was developed and prevalence of these mutations was investigated. The specific mutations were found in 15 (18%) of 88, five (28.6%) of 14, and four (67%) of six kanamycin, amikacin, and capreomycin-resistant *M. tuberculosis* isolates, respectively. All isolates carrying the mutations in the *rrs* gene were resistant to all three drugs. Therefore, the PCR-based technique should be useful for the fast prediction of cross resistance to the aminoglycoside antibiotics that are used for treatment of multidrug-resistant tuberculosis.

To investigate molecular nature of *M. tuberculosis* resistance to ofloxacin a spectrum of the mutations in the *gyrA* gene was determined by direct sequencing of 50 drug-resistant clinical isolates. Seventy percents of them carried mutations associated with resistance. The mutations observed among the *M. tuberculosis* isolates were consistent with distribution tendencies of mutations observed among isolates from the different countries. Therefore, genotypic drug susceptibility assays based on identification of specific mutations in *gyrA* gene should be effective diagnostic tools for rapid screening of fluorochinolones-resistant *M. tuberculosis*.

This work was supported by the Lithuanian State Science and Studies Foundation (projects T-43/04, T-56/05, C-20/2005.

Epigenetic Variation in Human Germ Cells

This work was performed in collaboration with The Krembil Family Epigenetics Laboratory (University of Toronto).

Phenotypic differences between individuals have traditionally been attributed to genetic (DNA sequence) variation and environmental differences. Numerous major international projects – from the Human Genome sequencing to creation of single nucleotide polymorphisms (SNP) databases and the Haplotype Map – have contributed significantly to the understanding of the position, degree, and structure of DNA polymorphisms. However, SNPs and other DNA sequence differences are relatively rare, and DNA sequences of two unrelated individuals are 99.5% identical. The objective of this study was to perform a comprehensive analysis of DNA methylation variation between- and within- the germlines of normal males.

First (in Institute of Biotechnology, Vilnius), methylated cytosines were mapped using bisulphite modification-based sequen-

cing in the promoter regions of the following disease genes: presenilins (*PSEN1*, *PSEN2*), breast cancer (*BRCA1*, *BRCA2*), myotonic dystrophy (*DM1*), and Huntington disease (*HD*). Major epigenetic variation was detected within samples as the overwhelming majority of sperm cells of the same individual exhibited unique DNA methylation profiles. In the inter-individual analysis, 41 of 61 pairwise comparisons revealed distinct DNA methylation profiles ($p = 0.036 – 6.8$ E-14).

Second (in The Krembil Family Epigenetics Laboratory, Toronto), a microarray-based epigenetic profiling of the same sperm samples was performed using a 12,198 feature CpG island microarray. The microarray analysis has identified numerous DNA methylation variable positions in the germ cell genome.

There are several reasons to believe that the germline may contain substantial epigenetic variation. Epigenetic reprogramming during gametogenesis, fertilization, and embryogenesis involves dramatic chromatin remodelling. Methylation reprogramming during gametogenesis involves the erasure and reestablishment of methylation of imprinted genes and other non-imprinted genes.

Results:

1. DNA methylation analysis of germ cells revealed intra- and inter- individual DNA methylation differences in the promoters of BRCA1, BRCA2, HD, DM1, PSEN1, and PSEN2.
2. This was confirmed by the analysis of the degree of uniqueness of DNA methylation profiles. In the case of HD, about 80% of all clones exhibited unique patterns of ^{met}C distribution.
3. While the intra-individual analysis can show variability within an individual, significantly variable methylation patterns between individuals were also revealed.
4. Male germline exhibits locus-, cell-, and age-dependent DNA methylation differences. These findings are interesting from both basic molecular biology and biomedical points of view.

This work was supported by the University of Toronto (Canada) and the Lithuanian State Science and Studies Foundation (projects T-42/04, T-55/05).

Grants

EU Framework 6 Programme
Lithuanian State Science and Studies Foundation
NorFa network
University of Toronto

Contracts

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UAB Grida (Vilnius, Lithuania)
Customs laboratory (Vilnius, Lithuania)
AB Kalnapilis-Tauras (Vilnius, Lithuania)

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Dr. R. Ulrich, Friedrich-Loeffler Institute, Greifswald, Germany

Publications 2004-2005

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11. Gedvilaite A., Zvirbliene A., Staniulis J., Kruger D.H., Ulrich R. Segments of puumala hantavirus nucleocapsid protein inserted into chimeric polyomavirus-derived virus-like particles induce a strong immune response in mice. *Viral Immunol*. 2004, 17, 51-68.
12. Butrimiene I., Jarmalaite S., Ranceva J., Venalis A., Jasiuleviciute L., Zvirbliene A. Different cytokine profiles in patients with chronic and acute reactive arthritis. *Rheumatology*, 2004, 43, 1300-1304.

Patent application

Zvirbliene A., Gedvilaite A., Ulrich R., Sasnauskas K.. A mean to produce monoclonal antibodies; hybridoma cell lines generated by this mean, and recombinant chimeric virus-like particles with inserted foreign protein fragments as immunogens to generate hybridomas by this mean. *1013 IP, 2005 043* (LT).

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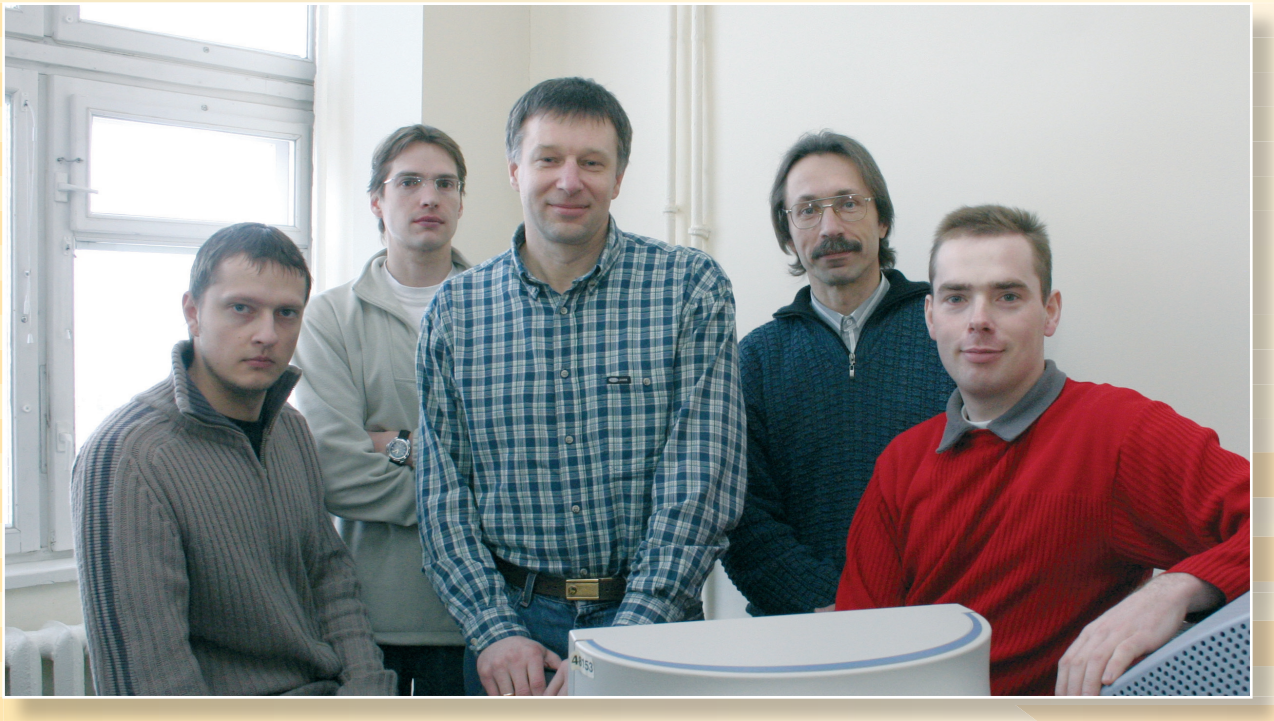


Albertas Timinskas, Ph. D.
Mindaugas Margelevičius, Ph. D.
Alvydas Špokas, M. Sc.
Rytis Dičiūnas, B. Sc.

In the post-genomics era bioinformatics is playing an increasingly important role in biological research. Breakthroughs in technologies have resulted in a flood of variuos types of biological data such as genome sequences for different organisms, data on gene expression, protein-protein interactions, etc. Bioinformatics is helping to make sense of all this vast biological data by providing tools that enable to perform large-scale analysis. In addition, bioinformaticians are utilizing available experimental data to improve various analytical and predictive methods that could help address specific biological problems.

Our newly established Bioinformatics laboratory is mainly focused on two research directions:

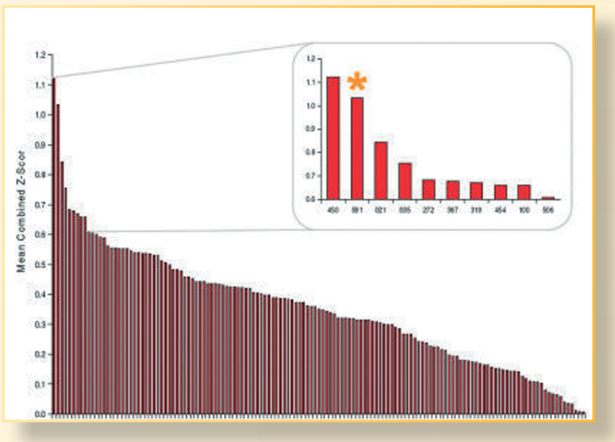
- Development of methods for protein modeling, assessment and analysis of protein structure and sequence comparison.
- Application of computational methods to characterize structure for both individual proteins and their complexes/interactions. Our main focus are DNA-interacting proteins, in particular those functioning in DNA replication, repair, recombination.

Development and testing comparative protein modeling methods

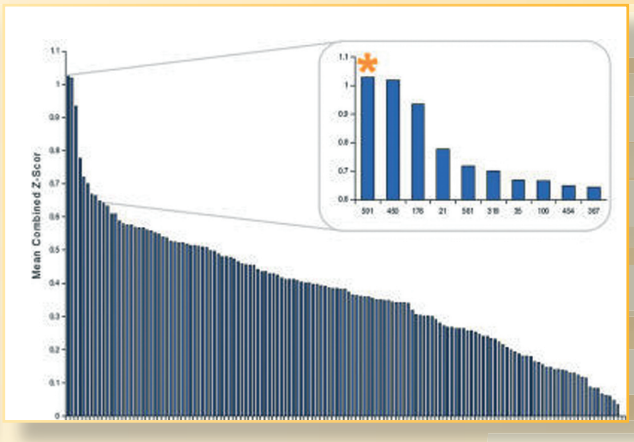
Development of protein modeling methods is an important long-standing area of structural bioinformatics. As individual researchers and most recently as a team (Bioinformatics laboratory) we have been actively involved in the development and testing of new protein modeling techniques based on homology. Our ideas for improving homology modeling approach to a large degree have been shaped by results of several rounds of a world-wide "blind" testing of protein structure prediction methods. This biennial experiment known as CASP (***C***ritical ***A***ssessment of ***S***tructure ***P***rediction) has been around for the last decade and has greatly stimulated progress in the field of protein modeling. The stimulating effect of CASP experiments is in that they provide a possibility to objectively test and compare different modeling approaches at the same time frame on the same set of target proteins, for which structures are about to be determined.

We have used CASP as testing grounds for our own comparative modeling approach as well as to analyze the overall improvements of protein modeling methods. CASP has established early on that the sequence-structure alignment is one of the major bottlenecks in improving model quality. Therefore, most of our developments have aimed at detecting unreliably aligned regions and at improved ability to choose the correct sequence-structure alignment for such regions. An independent assessment of CASP6 results has shown that our group is one of the world's leaders in homology modeling. Among over 150 groups evaluated in CASP6 homology modeling section, the overall results of our group were rated as the second best while the accuracy of the sequence-structure mapping (alignments) in our models was the best (see Fig).

Our modeling approach features a combination of both sequence- and structure-based methods. Initially, sequence-struc-



Rankings by GDT-TS z-scores



Rankings by AlO (alignment accuracy) z-scores

✱ - indicates results of the group (591-VENCLOVAS), representing Bioinformatics laboratory. The figure is adopted from the independent assessment report (Tress et al., *Proteins* 2005; Suppl 7:27-45.)

ture alignment is assessed in a position-specific way thus identifying suspect regions. These regions are then further evaluated within the context of a generated three-dimensional (3D) model. Recently, we have implemented the intermediate sequence search procedure (PSI-BLAST-ISS) for alignment reliability assessment as a freely available standalone software (Margelevičius & Venclovas, 2005; http://www.ibt.lt/bioinformatics/iss/). The detailed description and discussion of our overall CASP6 modeling results has been presented in an invited paper in the special CASP6 issue of *Proteins* (Venclovas & Margelevičius, 2005).

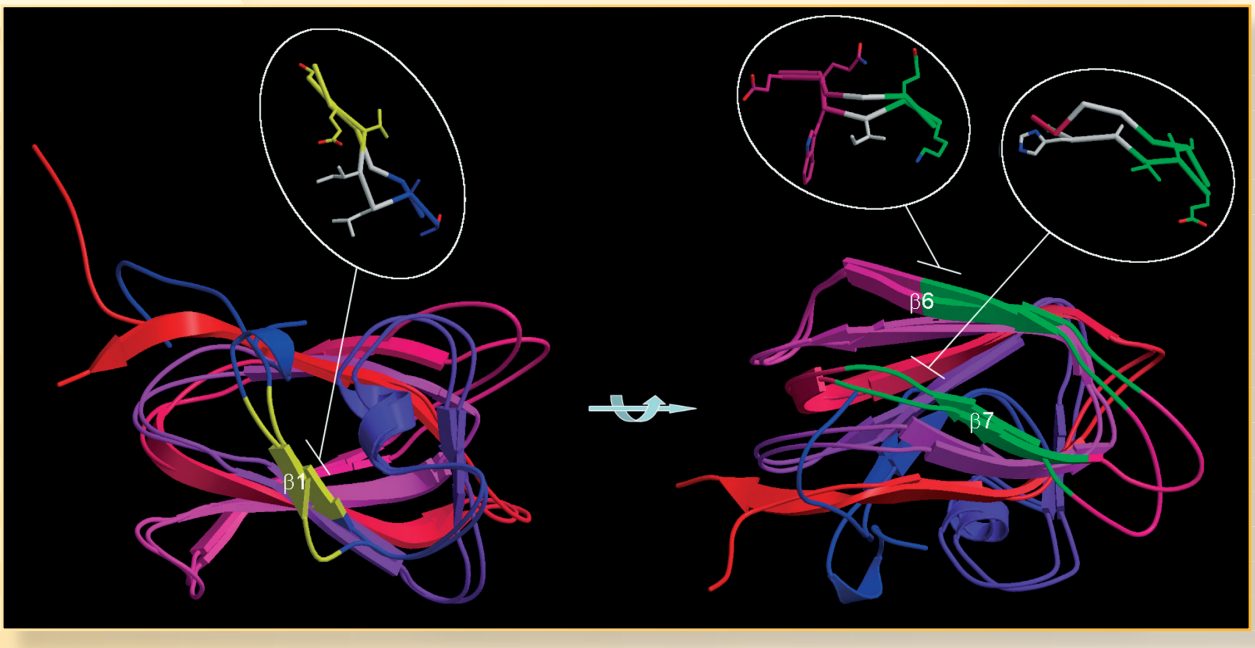


Figure. One of our most successful CASP6 predictions. The superimposed structures of the target (T0211) and our model are shown in two orientations. The coloring follows the progression of protein chain from N- (blue) to the C-terminus (red). For this protein we correctly modeled most of the chain including green regions where majority of other models were misaligned. Even with a small alignment error (yellow region), the fraction of correctly aligned residues in our model turned out to be significantly larger than in any other CASP6 model for this protein.

In addition to analysis of our own modeling results, we have been involved in an in-depth analysis of protein modeling field in general over the past decade (Kryshtafovych et al, 2005). This analysis has been performed in the context of CASP, since it is representative of the state-of-art in protein structure prediction. The results of this analysis have shown that there has been a modest improvement between the last two CASP experiments. On the other hand, the overall progress in protein modeling over the decade is impressive. The biggest leap has been made in distant homology modeling and fold recognition, where scores during this period have roughly doubled. Protein models are routinely produced of sufficient quality to design biological experiments at the level of individual residues such as site-directed mutagenesis. Along with the successes, our analysis has revealed a number of bottlenecks to progress such as the lack of effective structure refinement methods in high homology modeling and inability to reliably discriminate good models from bad ones in the template-free (new fold) modeling. We hope that these findings will help modeling community as a whole to concentrate methods development efforts most effectively.

Analysis and evaluation of protein structure quality

Success in protein modeling to a large degree depends on the ability to identify and correct errors in protein structure. One of the most detrimental yet often undetectable errors is the incorrect mapping of the sequence onto the structurally conserved region of the chain. Although infrequently, errors of this kind are sometimes inadvertently introduced even into protein crystal structures due to a low resolution or incomplete data.

We are continuously striving to find new more effective ways to evaluate protein structures, especially for the correctness of sequence-structure mapping. Recently, for this purpose we have developed a procedure that combines local energy assessment with our sequence alignment evaluator (Venclovas et al., 2004). To find out how effective it is, we have analyzed all Protein Data Bank crystal structures possessing one of the most populous folds, OB (oligonucleotide/oligosaccharide binding)-fold. We have detected at least five instances of sequence incorrectly mapped onto the structure. Moreover, we used results of computational analysis to direct a revision of X-ray structure for one of the entries having fairly inconspicuous error. Notably, both incorrect and revised structures appeared to be of similar quality by standard crystallographic parameters (R and R_{free} factors), but our computational analysis could clearly identify the correct vs. incorrect sequence-structure mapping. Based on our findings we suggest that such computational analysis may be useful not only for protein modeling, but may also facilitate crystal structure determination. In the course of structure determination it can help to guide the sequence-structure assignment process or verify the sequence mapping within poorly defined regions.

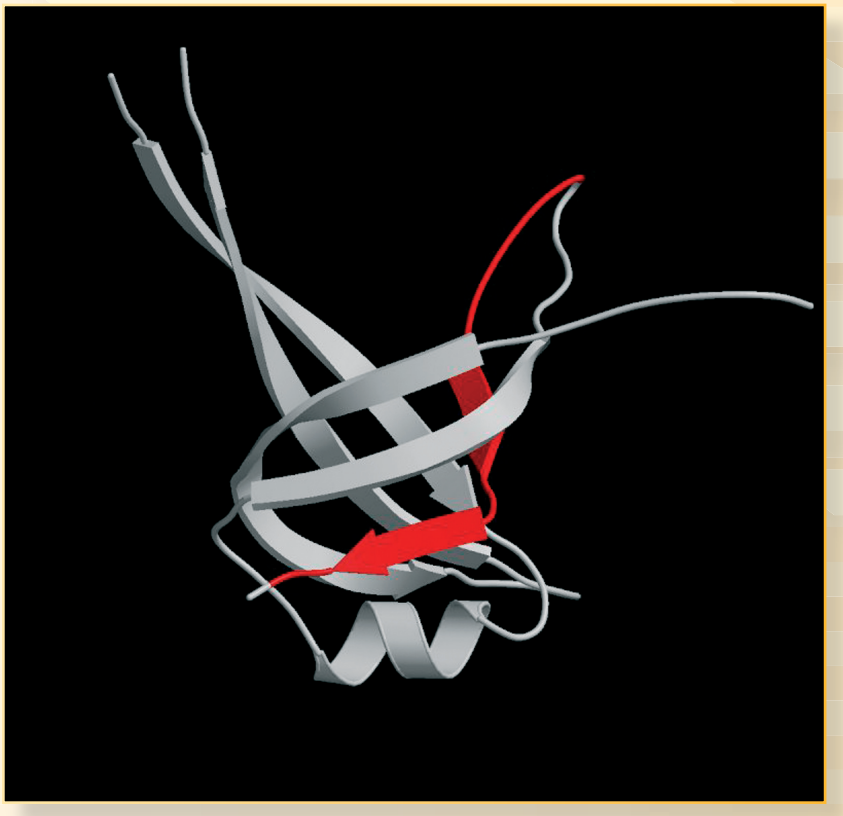


Figure. Crystal structure of human mitochondrial SSB protein solved at 2.4Å resolution. Incorrectly mapped sequence region is shown in red. Original flawed structure (3ULL) has R and R_{free} factors of 19.5% and 23.7% respectively. The corrected structure (1S30) has only slightly better R and R_{free} factors (19.1% and 23.0% respectively).

Application of molecular modeling to study proteins involved in DNA metabolism

In addition to methods development our laboratory is extensively using molecular modeling to help structurally and functionally characterize both individual proteins and their complexes.

Base-flipping initiation by DNA methylase HhaI

In a recent collaboration with Prof. Klimašauskas our aim was to establish early steps of the DNA methylation by the methyltransferase HhaI. The computational model of methyltransferase-DNA interaction has been instrumental in generating the hypothesis that the enzyme is actively initiating flipping of its target cytosine out of DNA helix. The results of this combined computational and experimental study confirmed the initial hypothesis contributing significantly to understanding of initial steps of a base-flipping phenomenon, which is utilized by many enzymes performing work on DNA (Daujotytė et al. 2004).

Mapping human MutY homolog (MutY) interaction with the DNA damage checkpoint complex

Another combined experimental-computational study was directed at characterizing interactions by the human MutY homolog (MYH), a DNA repair enzyme (collaboration with Dr. Lu, Maryland University, USA; paper in preparation). MYH reduces G:C to T:A mutations by removing adenines or 2-hydroxyadenines mispaired during DNA replication with guanines or 8-oxoG. In this study it was shown that MYH interacts with the DNA damage Rad9/Rad1/Hus1 checkpoint complex (known as the 9-1-1 complex). The 9-1-1 complex is a heterotrimer that presumably functions as a DNA damage sensor and is related to proliferating cell nuclear antigen (PCNA), a DNA sliding clamp. The interaction between MYH and the 9-1-1 complex was found to be mediated mainly through Hus1. Since MYH also interacts with PCNA, initially it was thought that the same MYH region is involved in interaction with both PCNA and the 9-1-1 complex. However, it turned out not to be so. To find out which other MYH regions could be responsible for interaction with the 9-1-1 complex we employed molecular modeling. First, we have modeled MYH structure, the 9-1-1 complex and DNA individually. Next, using these molecular models we explored structurally feasible ways of MYH – 9-1-1 interaction. As a result, we identified a putative binding region in MYH, which was then confirmed experimentally. Furthermore, computational analysis enabled us to narrow down the interaction to a few specific residues. Site-directed mutagenesis has indeed revealed their importance in mediating the intermolecular interaction. This study explains the physical basis for the observed MYH stimulation by the 9-1-1 complex and also is the first example of defining the 9-1-1 binding motif.

Collaboration

Dr. Daniel Barsky, Lawrence Livermore National Laboratory, Livermore, CA
Dr. Penny Beuning, Northeastern University, Boston, MA
Dr. A-Lien Lu, University of Maryland, Baltimore, MD
Prof. John Moult, University of Maryland Biotechnology Institute, Rockville, MD

Grants

Howard Hughes Medical Institute
EC Framework 6 Programme

Publications 2004-2005

1. Margelevičius M. & Venclovas Č. (2005) PSI-BLAST-ISS: an intermediate sequence search tool for estimation of position-specific alignment reliability. *BMC Bioinformatics*, 6:185.
2. Venclovas Č. & Margelevičius M. (2005) Comparative modeling in CASP6 using consensus approach to template selection, sequence-structure alignment and structure assessment. *Proteins*, 61 Suppl 7:99-105.
3. Kryshtafovych A., Venclovas Č., Fidelis K., Moult J. (2005) Progress over the first decade of CASP experiments. *Proteins*, 61 Suppl 7:225-36.
4. Barsky D. & Venclovas Č. (2005) DNA sliding clamps: just the right twist to load onto DNA. *Curr Biol.* 15:R989-R992.
5. Daujotytė D., Serva S., Vilkaitis G., Merkienė E., Venclovas Č., Klimašauskas S. (2004) HhaI DNA methyltransferase uses the protruding Gln237 for active flipping of its target cytosine. *Structure,* 12:1047-1055.
6. Venclovas Č., Ginalski K., Kang C. (2004) Sequence-structure mapping errors in the PDB: OB-fold domains. *Protein Sci.* 13:1594-1602.

Structure and Management of Institute of Biotechnology

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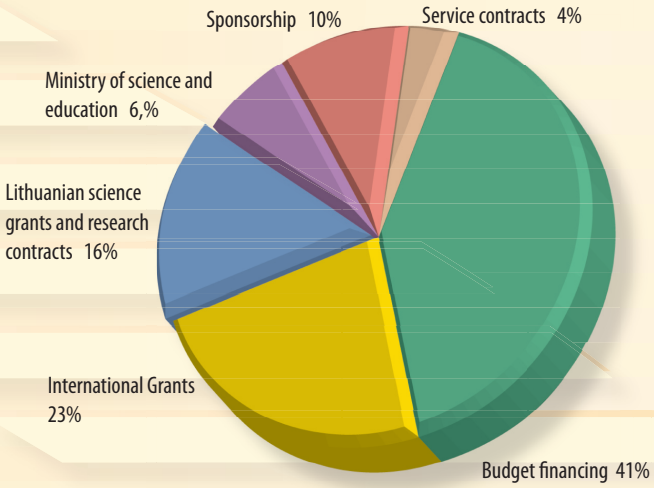
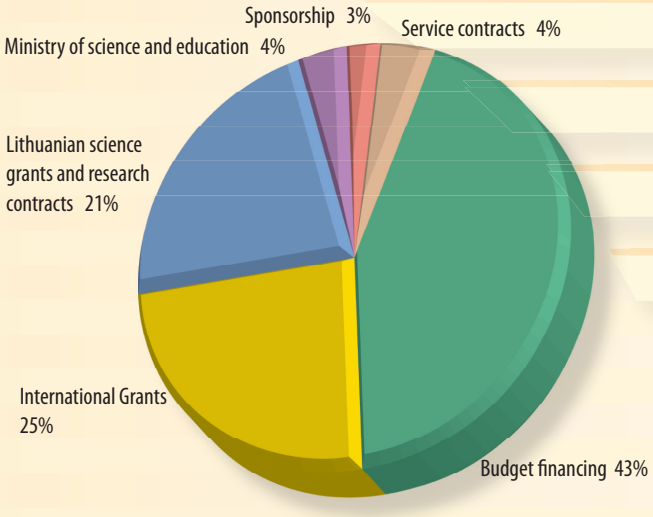
Financing sources, 2004

	Lt	EUR
Budget financing	2820700	816931
International Grants	1603382	464372
Lithuanian science grants and research contracts	1063836	308108
Ministry of science and education	442600	128186
Sponsorship	682061	197539
Service contracts	249604	72290
Total:	**6862183**	**1987426**



Financing sources, 2005

	Lt	EUR
Budget financing	3046000	882183
International Grants	1747837	506209
Lithuanian science grants and research contracts	1497870	433813
Ministry of science and education	264615	76638
Sponsorship	176100	51002
Service contracts	275450	79776
Total:	**7007872**	**2029621**
EU structural funds	**2917954**	**845098**



EUROPEAN UNION GRANTS

FRAMEWORK 6 PROGRAMME

Nr.	Project title	Project dates
1.	Rational design and comparative evaluation of novel geneticvaccines	2004 - 2008
2.	Targeting newly discovered oxygen sensing cascades for novel cancer treatments, biology equipment, drugs	2004 - 2008
3.	Drug design by structural thermodynamics	2004 - 2006
4.	Molecular modeling-based characterization of protein complexes involved in DNA repair	2004 - 2006
5.	ScanBalt Competence Region-a model case to enhance European competitiveness in life sciences, genomics and biotechnology for health on a global scale	2004 - 2006
6.	A multidisciplinary approach to the study of DNA enzymes down to the single molecule level.	2005 - 2009

FRAMEWORK 5 PROGRAMME

Nr.	Project title	Project dates
1.	Combined immune and gene therapy for chronic hepatitis	2001 - 2005
2.	Enhanced laboratory surveillance of measles	2002 - 2005
3.	Development of highly special enzyme for genome manipulation	2001 -2005
4.	Support for the integration of "newly associated states" NAS into the European research area	2003 -2006

EUROPEAN STRUCTURAL FUNDS

Nr.	Project title	Project dates
1.	Biotechnological agriculture and forestry plant research network	2005-2006
2.	The highest quality preparation of master and doctorial students in the fields agriculture and forestry biotechnology	2005-2007
3.	Improvement in the quality of human resourses in the research field of biotechnology	2005-2007
4.	Strengthening the experimental research material base in the interdisciplinary fields of biotechnology and other related fields	2005-2006
5.	Improvement of scientists qualification in the field of biotechnology and other related fields	2005-2006

Project manager, Vida Zemaitaitis with U.S. Secretary of State, Condoleezza Rice during her visit to Vilnius.

INTERNATIONAL GRANTS

HOWARD HUGHES MEDICAL INSTITUTE

Nr.	Project title	Project dates
1.	Combination of improved methods with expert knowledge to derive models of protein structures at low sequence homology	2001-2005
2.	Sequence recognition and base flipping by DNR metyltransferases	2001-2005
3.	Principles of restriction enzymes specificity	2001-2005
4.	Structural characterization of protein interactions in DNA replication, repair and recombination processes through molecular modeling	2006-2010

OTHER INTERNATIONAL GRANTS

Nr.	Project title	Project dates
1.	Max Planck Society - Crystallographic studies of restriction enzymes	2002-2005
2.	BMBF - Sequence-specific methyltransferase-catalysed labeling of biopolymers	2004-2005
3.	The Royal Society, European Science Exchange Programme- Correlating structure and spectroscopy	2002-2004
4.	NATO- Investigation of protein refolding factors and their implementation into biotechnical process	2002-2004
5.	Wellcome Trust - Cross-talk between functional domains of BfiI restriction endonuclease	2006-2007

NATIONAL GRANTS

HIGH-TECHNOLOGY DEVELOPMENT PROGRAMME

Nr.	Project title	Project dates
1.	New molecular tools for biotechnology	2003-2006
2.	Enhanced surveillance of respiratory viruses	2003-2006
3.	Generation of new monoclonal antibodies directed to desired epitopes using chimeric virus-like particles	2005-2006

Theses defended at the Institute of Biotechnology in 2004-2005

2004

G. Sasnauskas. Novel Subtype of Type IIs Restriction Enzymes. Scientific Supervisor: Prof. V. Šikšnys.

R. Rimšelienė. Construction of Restriction Endonuclease Eco57I Mutantas with Altered Sequence Specificity. Scientific Consultant: Prof. A. Janulaitis.

E. Mištinienė. Structure and Properties of Tumour Associated Antigen UK114 and its Homologue Protein p14.5. Scientific Consultants: Dr. V. Naktinis, Prof. G. Dienys.

2005

M. Zaveckas. Partitioning and Refolding of Recombinant Human Granulocyte-Colony Stimulating Factor in Aqueous Two-phase Systems Containing Chelated Metal Ions. Scientific Consultants: Dr. D. Matulis, Dr. H. Pesliakas.