DEPARTMENT OF MOLECULAR AND BIOMEDICAL SCIENCES

The research program of the Department of Molecular and Biomedical Sciences is focused mainly on basic research in protein biochemistry, molecular and cellular biology, and genetics. The primary goal of our investigations is the acquisition of new understanding of mammalian pathophysiology with the aim of improving human and animal health.

Secreted phospholipases A_2 ($sPLA_2$s)

The major research topic of the department are $sPLA_2$s originating from animal toxins as well as those found in humans. We are studying molecular mechanisms of action of toxic $sPLA_2$s, particularly those endowed with presynaptic neurotoxicity, and the role of endogenous $sPLA_2$s in pathological and physiological processes in mammals.

After we successfully demonstrated the translocation of ammodytoxin (Atx), a presynaptically neurotoxic $sPLA_2$ from the nose-horned viper (*Vipera ammodytes ammodytes*) venom, into the cytosol of a nerve cell, we made in the last year another step forward with the analysis of stabilization and the enzymatic activity augmentation of Atx in this cellular environment (L. Kovačič et al., *Protein Engineering, Design & Selection*, 23 (2010), 479–487). We found that, for the stabilization as well as for the potentiation of enzymatic activity of Atx in the reducing environment of the cytosol, the interaction of Atx with calmodulin (CaM), a protein in the cytosol of eukaryotic cells, is crucial. By measuring the binding affinity between different mutants of AtxA and CaM, and mapping of the interaction surface between Atx and CaM in the complex using a photoreactive derivative of AtxC, we set the constraints for the computer modelling of a structural model of the complex between these two molecules. The energetically most stable structural model of the AtxA–CaM complex is displayed in two different representations in Figure 1.

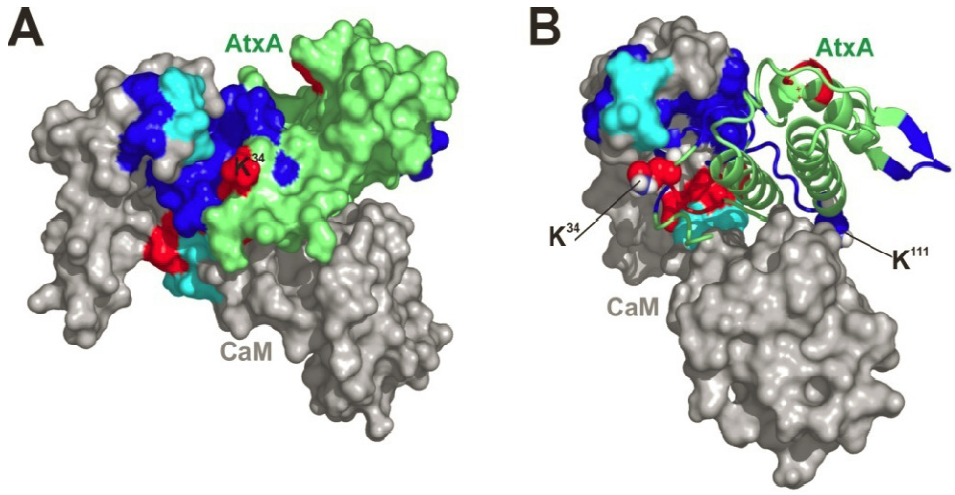

Figure 1. Structural model of the AtxA–CaM complex. From the model it is evident that CaM (gray) 'clamps' AtxA (green) between its N- and C-terminal domains, which is the most likely reason for the stabilization of AtxA in the complex. Regions that we identified at the rim of the interaction surface of both molecules are coloured blue, turquoise and red. The AtxA molecule is shown in the space-filling mode (A) and in the ribbon mode (B). In Figure 1B, Lys34 and Lys111 are labelled, being the most likely candidates to carry the photoreactive group in the Atx derivative. The figure is reproduced from the Doctoral Thesis of L. Kovačič (University of Ljubljana, 2010).

We verified the relevance of the model in a way that we exchanged the coordinates of AtxA in the complex for the coordinates of the structurally related $sPLA_2$s of either snake or mammalian origin and, wherever possible, generated structural model of a complex by computer modelling. Then we experimentally examined if the complex between each of the studied $sPLA_2$ and CaM was formed or not. In addition, we measured the ratio of initial enzymatic activity for each $sPLA_2$ in the presence and absence of CaM. The characteristics of $sPLA_2$s, which had been predicted from the $sPLA_2$–CaM modelling, were in all cases in accordance with the experimentally obtained data, confirming the correctness of our model. The AtxA–CaM structural model clearly shows why AtxA bound to CaM remains completely stable in reducing conditions in spite of the fact that it contains as many as seven intramolecular disulphide bonds. It shows also that the entrance to the active site of $sPLA_2$ is not restricted in the complex and that the surface area of the complex contacting the membrane is larger than that of the $sPLA_2$ alone, which nicely explains the increase in enzymatic activity of $sPLA_2$, either in reducing or non-reducing conditions. With the model, we thus provided a new tool to study the role of CaM in the process of $sPLA_2$ neurotoxicity. Even more importantly, by revealing the positive influence of CaM on the stability and enzymatic activity of certain endogenous $sPLA_2$s, such as group V an X $sPLA_2$s, we suggested a completely new way of regulation of these enzymes and processes dependent on their activity in the cytosol of mammalian cells. The significance of our discovery is best reflected by the fact that it was announced on the cover page of the issue publishing our paper (Figure 2).

Figure 2. Cover page of Issue 23 of the Protein Engineering, Design & Selection journal, decorated by the model of the AtxA–CaM complex. Important discovery of a possibility of the new mode of regulation of the enzymatic activity of $sPLA_2$s and all the processes related to this activity in the cytosol of mammalian cells with CaM has been announced on the cover page of the issue publishing our paper.

In collaboration with our colleagues from the Pasteur Institute in Paris, we determined three-dimensional structures of AtxA and AtxC by the means of protein crystallography and published the results (F.A. Saul et al., *Journal of Structural Biology,* 169 (2010), 360–369). The problem that we had been tackling for several years before we finally succeeded to solve it was the crystallization of these two toxic $sPLA_2$s. Comparison of the crystal structures of AtxA and AtxC revealed structural reasons for substantial difference in their enzymatic activity, neurotoxicity and anticoagulant action.

In 2010, we also continued with our research in the field of endogenous human $sPLA_2$s and their role in breast cancer. In humans, ten structurally different $sPLA_2$ enzymes are known that are involved in various physiological and pathophysiological processes: they participate in phospholipid digestion and homeostasis, in host defence against infections, influence the development of acute and chronic respiratory disorders, are important in lipoprotein remodelling and development of atherosclerosis, and, what has been observed particularly in recent times, they influence cell proliferation, apoptosis and cancer diseases. Namely, recent studies have

shown the correlation between expression of different $sPLA_2$s and pathological changes in stomach, colorectal and prostate cancer, with their roles in either tumour promotion or inhibition, depending on the tissue and biochemical microenvironment of the particular tumour involved. The role of $sPLA_2$s in breast cancer has not been studied to a considerable extent yet. There are, however, some earlier reports describing increased expression of the group IIA enzyme ($sPLA_2$-IIA) mainly in the more invasive parts of breast cancer tissues.

Our previous studies have also shown that $sPLA_2$s may stimulate or inhibit cell proliferation, induce cell death and are cytotoxic or neurotoxic, depending on the cell or tissue model system that we used. In our initial study of the involvement of $sPLA_2$s in the mechanisms that regulate the development and progression of breast cancer, we determined the expression profile of all $sPLA_2$ family members in seven human breast cancer cell models representing different stages in the progression of the disease. A validated method for quantitative PCR (qPCR) expression analysis of the whole set of human $sPLA_2$s in cells and tissue samples was set up through a collaboration with the PLA_2 laboratory from Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, France. In this way we identified the members of $sPLA_2$ family whose differential expression depends on the tumourigenic properties of the cell model that is used, indicating different roles of particular $sPLA_2$s at different levels of cancer progression. Further work in 2010 was focused on studying the impact of individual $sPLA_2$s on proliferation and invasion of breast cancer cells (Figures 3 and 4). To this end, we have selected among the identified group IIA, III, V and X $sPLA_2$s the latter ($sPLA_2$-X) as a priority. A gain-of-function study was performed by overexpressing $sPLA_2$-X in the selected cell line with highly invasive and tumourigenic properties and recombinant $sPLA_2$s were prepared to examine the effect of exogenously added $sPLA_2$s on breast cancer cells.

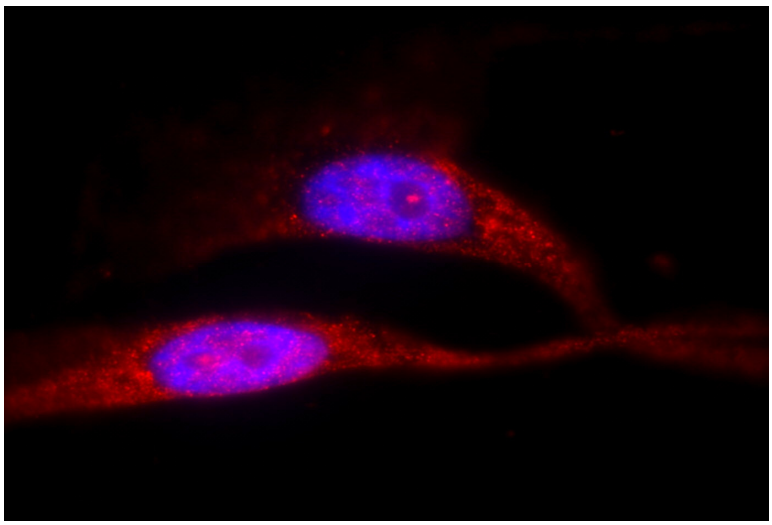

Figure 3. Observation of the localization of $sPLA_2$s in cancer cells by fluorescence microscopy. The cell nucleus is labelled blue.

By using the method for determining cell viability based on reduction of a methyl-thiazolyl-tetrazolium (MTT) dye and the proliferation assay based on the incorporation of a nucleoside analogue 5-ethenyl-2'-deoxyuridine (EdU) into the newly synthesized DNA of dividing cells, we found that ectopically expressed $sPLA_2$-X reduces viability and high proliferation rate of invasive cancer cells MDA-MB-231. We were also interested if the exogenously added $sPLA_2$ affected the growth of these cells since the most recent findings showed that extracellular and intracellular actions of$sPLA_2$s can be completely different. In our case, recombinant $sPLA_2$-IIA

and $sPLA_2$-X, which we successfully prepared in a bacterial expression system, effectively reduced the viability and proliferation rate when exogenously added to the highly invasive breast cancer cells. To obtain additional information on the proliferative properties of transfected cells, we used flow cytometry and a DNA dye (DAPI) to determine ploidy of the analyzed cells and their percentage in different phases of the cell cycle. Measurements were performed in the laboratory of flow cytometry at the Institute of Oncology, Ljubljana. The assays have shown that the cells overexpressing $sPLA_2$-X stopped their growth at the G_2/M stage of the cell cycle, which is consistent with the slower growth and morphological changes observed. Therefore, we will proceed with further analysis by flow cytometry to better understand the influence of $sPLA_2$-X on the cell cycle, cell death and cellular senescence. Our first studies on the role of $sPLA_2$s in breast cancer thus show that $sPLA_2$-X inhibits the cell proliferation rate, probably by causing an arrest in cell division at the G_2/M phase of the cell cycle, suggesting also a potential *in vivo* role of $sPLA_2$-X in the growth inhibition of breast cancer cells.

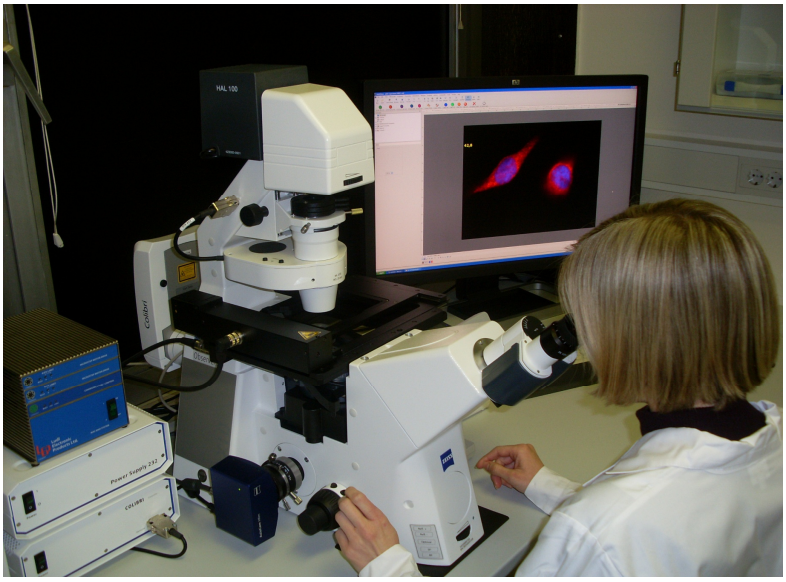

Figure 4. Work with a new epifluorescence microscope. The inverted fluorescence microscope Axio Observer (Zeiss) is a fully motorized system with two independent light sources, a conventional mercury lamp and Colibri LED light source system that supports a multiparameter analysis to monitor very fast processes. The microscope has an extreme sensitivity, with temporal and spatial resolution, which is essential for a high-performance subcellular fluorescent visualization of biological macromolecules in eukaryotic cells both in cell cultures and tissue preparations. It also offers monitoring of cellular processes such as cell cycle, cell signalling, integrity and changes in the membrane structures, intracellular localization of macromolecules and the interactions between macromolecules. The system will soon be upgraded with an incubator and modules that allow monitoring of processes in living cells.

We continued with the *in vitro* determination of chemotactic migration (invasiveness) of cells through the extracellular matrix, which allows us to assess the impact of $sPLA_2$s in the migratory and invasive abilities of cancer cells, which are key features in the formation of metastases. After careful optimization of the conditions of incubation and detection of cells in passing through the polycarbonate membrane, to which a basement membrane extract (Matrigel) was applied, we found that the cells expressing $sPLA_2$-X show a lower degree of invasiveness in comparison to unmodified cells MDA-MB-231. These results indicate that the expression of $sPLA_2$-X in a highly

invasive tumour tissue would impede the transition of cancer cells through the extracellular matrix and, consequently, reduce their invasiveness and consequently metastatic ability.

Our first results, aimed at studying the impact of $sPLA_2$s on the growth and invasiveness of breast cancer cells, are very promising and indicate the role of the phospholipase enzyme $sPLA_2$-X as a potential tumour suppressor. In the future, we would like to extend our studies to the mechanisms by which $sPLA_2$s affect proliferation and invasion of breast cancer cells, with an emphasis on determining the role of $sPLA_2$ enzymatic activity in those processes.

In the last year we published a procedure for the preparation and purification of human $sPLA_2$-X that enables obtaining larger, milligram quantities of the protein by a bacterial expression system (B. Jerman and J. Pungerčar, *Acta Chimica Slovenica*, 57 (2010), 888–894). Recombinant human group V and X $sPLA_2$s were added exogenously to a mouse motor neuron cell line and their influence monitored by various methods depending on the concentration of each $sPLA_2$ and differentiation of cells over time. The results were compared to the effects of better studied presynaptically neurotoxic $sPLA_2$s. We observed cytotoxic effects of both human $sPLA_2$s, with the largest influence a few hours after the addition. The effects of the two human $sPLA_2$s were similar to those of neurotoxic snake $sPLA_2$s, but being about 10-fold less toxic. To determine whether the cytotoxic effect is due to enzymatic activity and/or $sPLA_2$-binding to specific binding proteins, we developed an expression system for the production of two mutant human $sPLA_2$-X proteins, i.e. an enzymatically inactive (H48Q) and a cysteine (N79C) mutant. We assume that in this way it could be clarified whether exogenously added mammalian $sPLA_2$s also internalize into the cells of motor neurons, presumably in a similar way as some structurally related, neurotoxic snake $sPLA_2$s, and whether enzymatic activity is required for their cytotoxic action. The results will significantly contribute to the understanding of both endogenous as well as snake $sPLA_2$s in the (peripheral) nervous system.

In the scope of a bilateral project with the researchers from the Institute of Immunology in Zagreb, Croatia, we were optimizing the procedure for preparation of antiserum for treatment of the nose-horned viper envenomation. We found a high correlation between the quantity of Atx in venom samples and immunogenicity of these samples (B. Halassy et al., *Comparative Biochemistry and Physiology, Part C*, 151 (2010), 455–460). The content of Atx in particular venom is therefore a good prognostic factor for the suitability of this venom for preparation of highly effective antivenom by animal immunisation.

Together with the colleagues from the Biotechnical Faculty of the University of Ljubljana we finalized and published in 2010 the isolation, structural and functional characterization of an $sPLA_2$ from the venom of the Northern Pacific sea anemone, *Urticina crassicornis* (Figure 5) (A. Raspotnik et al., *FEBS Journal*, 277 (2010), 2641–2653).

Figure 5. The Northern Pacific sea anemone, *Urticina crassicornis*, in whose venom we discovered a $sPLA_2$ with a unique structure.

Analysis of the complete amino acid sequence of this protein was forecasting the discovery of a new type of $sPLA_2$. Namely, this molecule has a unique cysteine pattern, different from all known $sPLA_2$s, which also means a unique disulphide bonding (Figure 6).

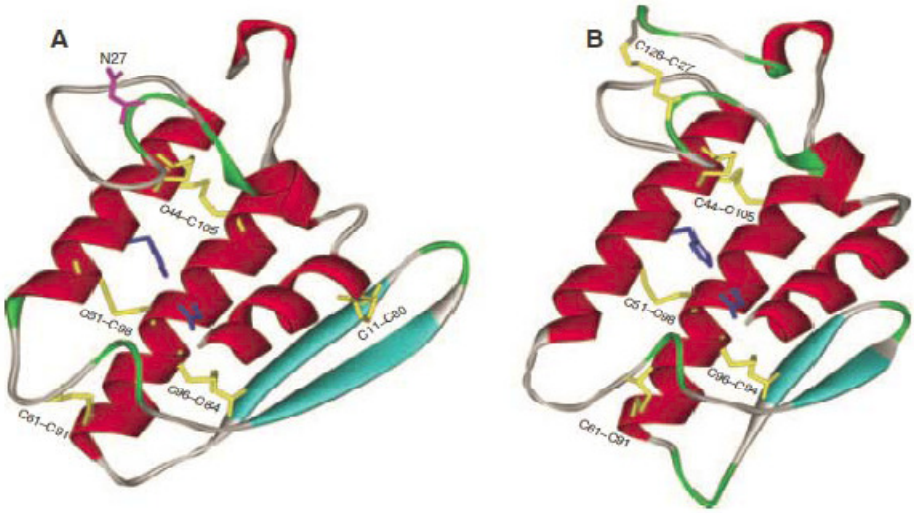

Figure 6. Structural models of $sPLA_2$s from see anemones *Urticina crassicornis* (A) and *Adamsia carciniopados* (B). The structural uniquenesses of *Uc* $sPLA_2$ in comparison to other group I $sPLA_2$s (*e.g. Ad* $sPLA_2$) are the substitution of the conserved Cys27 with Asn (purple N27) and shorter C-terminus of the molecule, therefore Cys126 is not present. Cys27 and Cys126 of the canonical group I $sPLA_2$s form a disulphide bond (labelled yellow) which restricts the flexibility of the C-terminal end of the molecule. As no such restraint is present in *Uc* $sPLA_2$, interesting biological features of this enzyme can be expected. The figure is reproduced from A. Raspotnik et al., *FEBS Journal*, 277 (2010), 2641–2653.

However, based on extensive analysis of the origin, distribution, diversity, evolution and classification of the metazoa-specific group I $sPLA_2$ family and Asn27 $sPLA_2$s we finally decided not to propose the introduction of a new group of $sPLA_2$s in the classification of these molecules. By searching all available metazoan genomic, proteomic and transcriptomic databases, we found that Asn27 $sPLA_2$s are not limited to the sea anemone *Urticina crassicornis*. The distribution of Asn27 $sPLA_2$s in metazoans is quite interesting, because they are present in diverse marine organisms (anthozoans, placozoans, mollusks and sea lampreys), in some freshwater organisms (crustaceans and planarians), and even in a few terrestrial invertebrates (centipedes and tardigrades). By phylogenomic analysis we demonstrated that group I $sPLA_2$s are present in all major metazoan taxonomic groups. Invertebrate group I $sPLA_2$s have undergone complex and dynamic evolution by numerous gene duplications (forming diverse multigene families), resulting in the greatest diversity of group I $sPLA_2$s in invertebrate genomes. Phylogenetic analysis provided evidence that, in invertebrates, a large number of species-specific multigene families evolved from a single ancestral group I $sPLA_2$ and became highly diversified by adaptive evolution, like group II $sPLA_2$s in snake venoms. Although orthologous relationships can be easily reconstructed for vertebrate group I $sPLA_2$s, our phylogenetic analysis failed to obtain any evidence for orthologous groups within invertebrate group I $sPLA_2$s. Nevertheless, due to its structural uniqueness, the $sPLA_2$ from *Urticina crassicornis* remains extremely interesting for further functional studies. Its haemolytic and neurotoxic activity on human erythrocytes and murine neuromuscular junction was negative.

Other pharmacologically active components from natural toxins

In 2010 we also intensively studied the components of the nose-horned viper venom that affect the coagulation of blood – haemostasis. The venom was fractionated by the means of different chromatographic techniques. In collaboration with the colleagues from the University Medical Centre Ljubljana, Division of Pediatrics, we tested the influence of venom fractions on different components of haemostatic system. We were focused in particular on metalloproteinases (MPs) in the venom. We succeeded to determine most of the amino acid sequence of ammodytase, a fibrinolytic MP with the molecular mass of about 70 kDa, a potential antithrombotic agent. Based on the elements of the primary structure of ammodytase and related MPs we synthesized oligonucleotide probes to isolate their cDNAs from a snake venom gland cDNA library. We succeeded to amplify, clone and sequence cDNAs encoding several related MPs; however we are still trying to find that coding for ammodytase.

As one of the 20 partners on the EU 6FP integrated project "Conco" we have been involved in the analysis of the genome, transcriptome and venom proteome of the piscivorous marine snail *Conus consors* and related snails. In the past year we also continued to work on the preparation of a recombinant novel conotoxin, which presumably contains six intramolecular disulphide bonds, whose function and biological role are not yet known. We amplified the section encoding mature conotoxin in the appropriate cDNA, coding for a longer peptide precursor, and inserted it into an expression plasmid under the control of a strong promoter that enabled its expression in the form of inclusion bodies in the cytoplasm of bacterial cells. Currently, we are focused on the isolation and attempted renaturation of insoluble and incorrectly folded recombinant conotoxin from bacterial cells. We also continued the proteomic analysis of the *Conus consors* venom in the range of molecular masses exceeding 10 kDa and identified many new components. We performed phylogenetic and biogeographic analyses of diverse Indo-Pacific and endemic South African venomous cone snails (S. Kauferstein et al., *Toxicon*, accepted for publication). A phylogenetic analysis of the 16S RNA from numerous *Conus* species collected in the South Africa and deep sea waters of New Caledonia, Marquesas, Tahiti, Takapoto Island and Philippines has been made. The 16S RNA phylogenetic analysis confirmed that *Conasprella* represents the most basal lineage of *Conus* species and that the endemic South African *Conus* snails belong to a few *Conus* clades only. They most likely originated from Indo-Pacific, but not from the West Atlantic species. Widely distributed *Conus* species disperse through planktonic larvae, while endemic species reproduce with non-planktonic larvae. It is apparent that endemic South African *Conus* species originated from Indo-Pacific species after the shift in the mode of their development. Species evolution and geographic spreading may have been hindered by ecological constraints such as the effect of ocean currents, *e.g.* the Agulhas current. These peculiar ecological conditions may constitute barriers preventing further spreading of local species and may favour neoendemism resulting in allopatric speciation.

High-throughput genetics and functional genomics in yeast *Saccharomyces cerevisiae*

In the future, genomic data will enable more accurate prediction of development of diseases and their treatment in humans, and advanced biotechnological processes. Inference of new and useful hypotheses from heterogeneous sources of genome-scale experimental data however requires new computational methods that can integrate different types of data. In collaboration with the Bioinformatics Laboratory of the Faculty of Computer and Information Science, University of Ljubljana, we developed an integrative data analysis approach, which, rather than correlating the findings from different types of data sets, uses each type of data independently to identify the components of molecular pathways and combines them into a single directed network. Our computational genomics approach is based on a set of inference rules traditionally used for reasoning on genetic experiments, which we have formalized and implemented in a software tool. With the approach using chemogenetic interaction and expression data and by using previous knowledge on the set of genes whose expression the transcription factor in

question regulates, we have successfully inferred the models for the action of the drug rapamycin and of a DNA damaging agent on their molecular targets and pathways in yeast cells. The developed method has been published in one of the leading journals in the field of functional genomics (M. Mattiazzi et al., *OMICS: a Journal of Integrative Biology*, 14 (2010), 357–367) and is available as a web-based tool at http://www.ailab.si/perturbagen.

Modulation of the composition and shape of biological membranes is an emerging mode of regulation of cellular processes. Phospholipases A_2 (PLA_2s), an extensively studied group of proteins at our department for many years, exert their biological activities through affecting both membrane composition and shape. The aim of the study, which was carried out in collaboration with a laboratory from the University of California at San Francisco, was to investigate the global effects that such perturbations have on a model eukaryotic cell. Our genome-wide analysis of cellular effects of a PLA_2 in yeast *Saccharomyces cerevisiae* demonstrated functional genetic and biochemical interactions between PLA_2 activity and the Rim101 signalling pathway. Our results suggested that the composition and/or the shape of the endosomal membrane affect the Rim101 pathway. We described a genetically and functionally related network, consisting of components of the Rim101 pathway and the prefoldin, retromer and SWR1 complexes, and predicted its functional relation to PLA_2 activity in a model eukaryotic cell. This published study (M. Mattiazzi et al., *Molecular Genetics and Genomics*, 283 (2010), 519–530) provided a list of the players involved in the global response to changes in membrane composition and shape in a model eukaryotic cell.

Evolutionary genomics of transposable elements and functional studies of retrotransposons

Transposable elements (TEs) have profound effects on the structure, function and evolution of their host genomes. Our knowledge about these agents of genomic change in sauropsids, a sister group of mammals that includes all extant reptiles and birds, was very limited. The large amount of recently accumulated genome-wide data on TEs in diverse lineages of sauropsids has provided a remarkable opportunity to review current knowledge about TEs of sauropsids in their genomic context (D. Kordiš, *Cytogenetic and Genome Research,* 127 (2009), 94–111 - published in 2010). Avian and reptilian genomes differ significantly in the classes of TEs present, their fractional representation in the genome and by the level of TE activity. In sauropsid genomes TEs have been active for hundreds of millions of years, and as such have had a huge impact on the overall architecture of the genomes, such as contracting or expanding the size of the genomes and providing regions of sequence identity for recombination events that could generate genetic diversity in reptilian genomes.

Other subjects

In 2010 we also collaborated at several projects out of the thematic scope of our department or the programme group "Toxins and biomembranes".

The methodology of preparation of the photoreactive derivative of AtxC was successfully used to prepare photoreactive derivatives of human procathepsin K to map the interaction surface between procathepsin K and heparin in order to build a structural model of the complex between these two molecules (M. Novinec et al., *Biochemical Journal,* 429 (2010), 379–389). By analysis of the DNA and RNA samples of patients with unipolar depression we participated at the pharmacogenetic study of alternative antidepressant response of these people (R. Uher et al., *American Journal of Psychiatry*, 167 (2010), 555–564; K. Malki et al., *Biological Psychiatry*, (2010), doi:10.1016/j.biopsych.2010.08.011). With structural analysis we participated at the identification of protein interactors of stefin B, an inhibitor of cysteine proteinases, in the nucleus of mammalian cells. We discovered that stefin B binds to nucleosome, specifically to histones H2A.Z, H2B and H3, in the nucleus of mammalian cells, which may be important for the occurrence of the Unverricht-Lundborg disease (S. Čeru et al., *Journal of Biological Chemistry*,

285 (2010), 10078–10086). We participated in unravelling the reason for anomalous cellular localization of TDP-43 protein in the case of amyotrophic lateral sclerosis and TDP-43 proteinopathies. In these diseases, TDP-43 localizes and aggregates in the cytosol rather than in the cell nucleus. We suggested the explanation for the improper translocation of TDP-43 (A.L. Nishimura et al., *Brain*, 133 (2010) 1763–1771).