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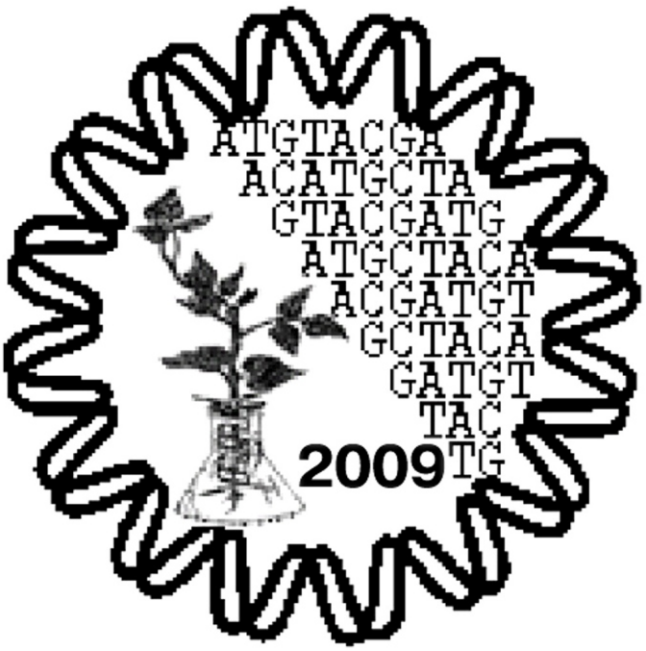

Materials of the Third Conference of Young Scientists, dedicated to Charles Darwin's 200th Birthday and the 150th Anniversary of the publication of his main book «On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life» (9 10 June 2009)

Collection of Theses

Expression and function of chitinase-3 like protein 1 (*CHI3L1)* in brain glial tumors

O. V. Balynska, A. V. Iershov, V. V. Dmitrenko, V. M. Kavsan

Institute of Molecular Biology and Genetics NAS of Ukraine

l.v.balynskaya@gmail.com

Aim. Chitinase-3 like protein 1 (CHI3L1, HC gp-39 or YKL-40) is a human cartilage glycoprotein encoded by *CHI3L1* gene revealed by us previously among the highly upregulated genes in glioblastoma – the most aggressive type of human brain tumors. Increased levels of circulating CHI3L1 have been reported in both patients with breast and colorectal cancer and patients with liver cirrhoses. In connective tissue cells CHI3L1 initiates signaling cascade which leads to increased cell proliferation. The objective of this project is to find possible involvement of CHI3L1 into the main cellular signaling pathways, mainly into MAPK and PI3K cascades in glial cells as well as to find possible CHI3L1 protein partners. **Methods**. cDNA CHI3L1 was cloned into *pET-24a* vector, expressed in *E. coli* cells, a recombinant protein was purified on Ni-NTA-agarose. U-87 MG and HEK-293 cells were grown about to the confluence in DMEM supplemented with 10 % FBS and 100 g/ml penicillin and 100 units/ml streptomycin in 6-well tissue-culture plates. Human embryonic kidney 293 (HEK-293) cells were serum-starved for 24 h, followed by exposure to MG-63 cell medium enriched with CHI3L1 or recombinant CHI3L1 for 1h. At the end of the incubation period, cell layers were washed twice with ice-cold PBS, lysed in SDS buffer, and the cell lysates were analysed by SDS/PAGE and Western blotting. The blots were exposed to the phosphorylation-specific antibodies at dilutions recommended by the manufacturer and reprobed with the pan-specific antibodies to determine total ERK1/2 protein. **Results**. The results obtained have shown that CHI3L1 plays a certain role in the mitogen-activated protein signaling cascade (MAPK) involved in the fibroblast mitogenic response. To test a possibility of CHI3L1 participation in the activation of extracellular signal-regulated protein kinases (ERK1/ERK2), we used the human embryonic kidney 293 (HEK-293) cell line, which like many other cell types, in monolayer culture grows in unsupplemented culture medium for no longer than one week loosing whereupon its viability. The results suggest that addition of CHI3L1 stimulates ERK1/ERK2 phosphorylation in these cells. No ERK1/ERK2 phosphorylation was observed either in the cells exposed to the medium without CHI3L1 or to the medium supplemented with BSA. In contrast to HEK-293, phosphorylation of ERK1/ERK2 could be seen in U-87 MG cells exposed to the medium without CHI3L1 addition or in cells exposed to the medium supplemented with BSA. The reason for this difference may be explained by our previous finding that U-87 MG cells produce CHI3L1. We cloned CHI3L1 cDNA into expression vector *pCMV*-flag N-terminal to search for potential CHI3L1 protein partners. The expression of recombinant protein was confirmed by transfection in mammalian cells and further Western blot analysis of the lysates. The search for partners of CHI3L1 with co-immunoprecipitation is the next step of our research. **Conclusions**. In this work we have found that CHI3L1 is involved in the activation of mitogen-activated cellular signaling pathway (MAPK) through phosphorylation of ERK1/ERK2 in human embryonic kidney cells and human glial cells.

Editing of errors by *Enterococcus faecalis* prolyl-tRNA synthetase

K. S. Boyarshin, I. A. Kriklivyi, A. D. Yaremchuk, M. A. Tukalo

Institute of Molecular Biology and Genetics NAS of Ukraine
kboyarshin@mail.ru

The two-stage mechanism, which includes a specific recognition of amino acids and editing of synthesized products, is required to maintain the amino acid specificity of different aminoacyl-tRNA-synthetases. The ARSases class II show much more diversity of editing mechanisms that are less investigated than those of the class I. Among them the least studied is prolyl-tRNA synthetase (ProRS), the spatial structure of which was resolved only recently. ProRS is able to activate not only proline but alanine and cysteine as well. The enzyme is known to accomplish pretransfer editing of alanine through hydrolysis of alanyl-adenylate, and posttransfer editing through hydrolysis of alanyl-tRNA that takes place in a particular editing domain. The aim of this work was to outline the ways of further study on the editing activities of prokaryotic ProRSs. Using a computer model and experimental data we have put forward several suggestions about the structure of ProRS editing domain active center and performed site-directed mutagenesis of the ProRS gene (alanine scanning). An essential role of three amino acid residues, K279, G331 and H366, was shown for alanyl-tRNA deacylation. One of the mutant forms, K279, almost completely lacks the posttransfer editing activity. However, this mutant form shows an essential increase of editing activity in ATP hydrolysis test in the presence of alanine under conditions of adding $tRNA^{Pro}$ to the reaction mix, that probably indicates the pretransfer editing induction by $tRNA^{Pro}$. This result is in controversy with those obtained for analogous enzyme from *Escherichia coli* which possesses only a weak tRNA-independent pretransfer editing activity, and it may indicate rather wide possibilities of pretransfer editing in some ProRS. Some peculiarities of tRNA structure have an influence on the effectiveness of the pretransfer editing induction. The hydroxyl groups of 2'- and 3'-adenosine ribose is an extremely important element of tRNA structure in the abovementioned context. By the methods of non cognate amino acid dependent ATP hydrolysis, we have also demonstrated the existence of tRNA-independent pretransfer editing of cysteine which is of great interest because the mechanisms of ProRSs cysteine discrimination are poorly investigated. Taken together, the results obtained considerably broaden our outlook on editing mechanisms of prokaryotic ProRSs and indicate the pretransfer editing against alanine and cysteine.

Coenzyme A Synthase influences activity of signal transduction pathways in mammalian cells, forms complexes with p85 regulatory subunit of PI3K and promotes cell transformation *in vitro*

O. S. Breus[1], **G. G. Panasyuk**[1, 2], **I. T. Gout**[2], **I. O. Nemazanyy**[1], **V. V. Filonenko**[1]

[1]Institute of Molecular Biology and Genetics NAS of Ukraine

[2]University College London, London, UK

oksanabreus@yahoo.com

Coenzyme A (CoA) and its derivatives play a complex role in cell metabolism and signalling. CoA Synthase (CoASy) is mitochondria associated enzyme which mediates two final stages of *de novo* CoA biosynthesis. The complex interplay between cellular signalling and metabolism just starts to emerge. Here, we report that CoASy is involved in signalling events in the cell by forming a complex with p85 PI3K and influencing activity of PI3K-dependent pathway *in vivo*. In pull down screening we have found that recombinant SH2 and SH3 domains of p85 regulatory subunit of PI3K precipitate endogenous CoASy from tissue and cellular extracts of rat organs and HEK293 cells. Further, existence of p85 -CoASy complex in HEK293 cells *in vivo* was confirmed in co-immunoprecipitation experiment. Interestingly, we detected a fraction of catalytic p110 and regulatory p85 subunits of PI3K in mitochondria fraction and have found that mitochondria associated p85 PI3K is in complex with CoASy. Surprisingly, the significant influence of cellular CoASy level on activity of PI3K signalling pathway was discovered in the experiments with siRNA mediated CoASy knockdown. Furthermore, the positive impact of CoASy on cell survival and anchorage independent cell growth was revealed. An ability of adherent cells to divide without contacts with extracellular matrix is a well known hallmark of their malignization, the reason of which is the hyper activation of mitogen- and integrin-signals transmitting pathways. Depletion of CoASy level by siRNA in human hepatocellular carcinoma cells (HepG2) led to significant decrease in its ability to form colonies in semisolid agarose, while CoASy overexpression in HEK293 cells led to up to two-fold enhancement of this ability. Importantly, that using overexpression of catalitically inactive or cytoplasm localized CoAsy mutants, we have demonstrated that CoASy activity and its association with mitochondria are crucially important for these effects. Collectively, our data represent a novel link between intracellular signalling and metabolism, and point on CoASy as a novel potential protooncogene.

Synthesis and optimization of 2-phenylisothiazolidin-3-one-1,1-dioxides as inhibitors of human protein kinase CK2

M. A. Chekanov, A. R. Synyugin, S. S. Lukashov, S. M. Yarmoluk

Institute of Molecular Biology and Genetics NAS of Ukraine
chekanov-maxx@ukr.net

Protein kinase CK2 (Casein Kinase 2) plays an important role in the transduction and enhancement of various cell growth and metabolic signals. The CK2 deregulation in various pathological processes suggests that CK2 inhibitors should have a therapeutic value, particularly as antineoplastic and antiviral drugs. The aim of our work was to synthesize new derivatives of 2-phenylisothiazolidin-3-one-1,1-dioxides and study their inhibition activity toward kinase CK2. Potential inhibition activity of these compounds was predicted by flexible docking (programm package DOCK 4.0). Synthesis of the predicted compound series was carried out using combinatorial synthesis techniques. The series of 19 compounds was tested *in vitro* for CK2 inhibitory activity. It was found that N-(3-acetylphenyl)-2-chloro-4-(4-methyl-1,1-dioxido-3-oxoisothiazolidin-2-yl)benzamide has IC_{50} value of 20 M. To perform further structure optimization, another 7 compounds of this class were synthesized. Five of them showed IC_{50} value less than 20 M. The most active compound is 4-{[2-chloro-4-(1,1-dioxido-3-oxoisothiazolidin-2-yl)benzoyl]amino}-2-hydroxybenzoic acid with IC_{50} value of 3 M. Thus, we suppose that the represented compounds are a promising class of novel CK2 inhibitors.

Latent membrane protein 2A (LMP2A) of Epstein-Barr virus interacts with endocytic adaptors: intersectin 1 and amphiphysin 1

O. V. Dergai, M. V. Dergai, I. Ya. Skrypkina , L. O. Tsyba, G. Winberg[1], A. V. Rynditch

Institute of Molecular Biology and Genetics NAS of Ukraine
[1]Karolinska Institute, Stockholm, Sweden
o.dergai@gmail.com

Aim. Endocytosis is a fundamental process of vesicle-dependent substrates uptake and intracellular trafficking. Importance and significance of this process are emphasized by the fact that numerous nutrients, growth factors, messengers and pathogens enter cells utilizing different types of endocytosis. Intersectin 1 is an endocytic adaptor protein known to be crucial at the earliest stages of endocytosis. Moreover, it controls RTK-signaling in concert with c-CBL, promotes actin cytoskeleton nucleation in Cdc42- and NWASP-dependent manner. Different pathogens use hosts mechanisms of endocytosis and signalling to enter cells and affect cell differentiation and proliferation. Epstein-Barr virus (EBV) infects epithelial cells and B-lymphocytes, in the latter the virus is life-long latent. Only restricted set of viral genes are expressed within the latent phase: LMP1, LMP2A, LMP2B, EBNAs and EBERs. LMP2A mRNA is frequently detected in peripheral blood B-lymphocytes from healthy individuals, and the protein is often present in tumor biopsies from EBV-associated malignancies. **Methods**. Molecular cloning techniques, site-directed mutagenesis, GST-pull down and immunoprecipitation assays, immunofluorescence analysis. **Results and Conclusions**. Here we report about interaction between viral protein LMP2A and endocytic adaptor intersectin 1.The immunoprecipitation data evidence to complex formation between LMP2A and ITSN1 *in vivo* in HEK293 cells and in B-lymphocyte cell line CBMI. SH3-domains of ITSN1 are sufficient to precipitate LMP2A *in vitro*, thus it was supposed that ITSN1 binds -PXXP- motives of LMP2A. Moreover, another endocytic adaptor – amphiphysin 1 has been found to bind -PXXP- of LMP2A through its SH3 domain. Mutational analysis of LMP2A sequence indicates at least two binding sites for ITSN1 and three – for amphiphysin. According to our data intersectin1 interacted with both isoforms: LMP2A and 2B, while amphiphysin bound only LMP2A. Amphiphysin 1 bound two sites at the N-terminus of LMP2A which were known to mediate the interaction of this viral protein with ubiquitin-ligases Nedd4-2 and Aip4. Thus, it is tempting to speculate that amphiphysin 1 competes with mentioned ubiqutin-ligases inhibiting downregulation of LMP2A associated proteins like Syk and Lyn, as well as LMP2A by itself. An analysis of subcellular distribution of LMP2A and ITSN1 in MCF7 cell line supported our data on these proteins interaction *in vivo*. Using immunofluorescence analysis we suggested that ITSN1 and LMP2A interact initially during LMP2A internalization on plasma membrane. Summarizing these data we can propose a model of clathrin-mediated internalization of LMP2A which could be ITSN1-dependent. Investigation of ITSN1–LMP2A interaction could provide an important clues for the understanding of LMP2A role in the infection and EBV-dependent cancerogenesis.

Single-chain antibody and alkaline phosphatase immunoconjugates engineered

A. I. Flyak, M. V. Tsapenko, I. M. Gilchuk[1], P. V. Gilchuk[1]

Institute of Molecular Biology and Genetics NAS Ukraine
[1]Institute of Genetic and Regenerative Medicine of the AMS Ukraine
flyaka@mail.ru

Specific monoclonal antibodies conjugated with enzyme tags are valuable immunoreagents widely used in medical and laboratory practice for detection of target antigens. Traditionally, conjugation of antibodies with tags is achieved by chemical coupling, which requires relatively large quantities of purified antibodies, a chemically activated tag, and controlled conditions of the reaction. The complexity of the process of obtaining monoclonal antibodies with desired specificity, as well as the necessity of the chemical conjunction stage, altogether, make for the high cost of the resulting probes. Modern DNA cloning technologies allow generation of engineered monoclonal antibodies and their fusion with other protein partners. Hereafter, such fusion proteins will probably be produced in bacteria with the use of traditional fermentation schemes and affordable nutrient media. In view of this, generation of engineered conjugates by means of chimerization of recombinant antibodies with proteins demonstrating enzymatic activity seems a promising alternative to the traditional way of obtaining monoclonal antibody conjugates. One of the most perspective fusion partners is alkaline phosphatase (AP). There are a number of commercially available calorimetric, as well as fluorescent and chemoluminescent media for AP, which allow using different antigen detection schemes and provide high sensitivity of immunoassays. The goal of the present work was to generate fusion proteins based on a bacterial alkaline phosphatase (BAP) with enhanced catalytic activity and single-chain antibodies (ScFv's) against several antigens, as well as to estimate the applicability of the obtained immunoconjugates in different immunoassays. The following methods and techniques were used in this work: bacteria cells culturing, gene cloning, polymerase chain reaction, DNA electrophoresis, DNA sequencing, protein expression, ELISA, Western-blotting, immunocytochemistry, protein electrophoresis, estimation of enzymatic activity, microscopy. To obtain the enzyme with enhanced catalytic activity the mutagenesis of BAP was carried out. Using previously isolated genes of mouse ScFv's against hIFN-a2b, hIFN-b1b, hCD34 several of ScFv-BAP_{mut} fusion proteins were designed and recombinant plasmids for their expression in *E. coli* were created. All fusion proteins were produced in bacteria by secretion into the periplasm and culturing medium. The functional activity of both moieties of secreted ScFv-BAP_{mut} protein was shown. The applicability of such alkaline phosphatase engineered immunoconjugates in antigen detection by means of ELISA, immunoblotting and immunocytochemistry has been shown.

Identification of target sites of regulatory elements in 3' UTR of human intersectin 1 mRNA

D. O. Gerasymchuk, S. V. Kropyvko, L. O. Tsyba, A. V. Rynditch

Institute of Molecular Biology and Genetics NAS of Ukraine
dmitriy.gerasimchuk@gmail.com

Aim. Human intersectin 1 gene (ITSN1) encodes two isoforms (ITSN1-S and ITSN1-L) of multidomain adaptor protein that takes part in clathrin-mediated endocytosis, cell signaling and cytoskeleton reorganization. It is known that many genes are regulated posttranscriptionally by different factors that have targets in 3'UTRs of their mRNAs. The aim of our work was to identify full-length 3'UTR of ITSN1-L and define target sites for different factors (microRNA and proteins) that could regulate ITSN1 on posttranscriptional level. **Methods**. To define full-length 3'UTR of ITSN1-L we performed computational analysis of GenBank EST database. To confirm our results we performed 3'RACE, RT-PCR, cloning of RT-PCR products in pGEM-T-easy vector, and sequencing. To identify target sites of regulatory factors we used such bioinformatic web-servers as TargetScan.org ver. 2.1, ver. 3.1, and ver. 4.1, microRNA.org, mirGen.org, and Diana-microT for microRNA target prediction and ARED database for protein factors target sites prediction. We cloned 3'UTR of ITSN1-S cDNA and performed luciferase assay to validate received data. **Results**. While performing EST database analysis we identified 11 ESTs which showed that the most probable end of 3'UTR of ITSN1-L mRNA could be located 11559 downstream from the characterized 3' end of 41 exon. RT-PCR, 3'RACE, and sequencing confirmed these prediction. Computational analysis of 3'UTR of ITSN1-S mRNA identified putative target-sites for 15 different microRNAs. We also analyzed 3'UTRs of mRNAs of ITSN1 interacting partners and found 4 common target sites in 3'UTRs of ITSN1-S, dynamin-1, SNAP-23 and EPS-15. To confirm these findings we performed luciferase assay using the construct based on pTKluc vector with insertion of full-length 3'UTR of ITSN1-S. We found the 10-fold inhibiting of pTKluc-3'UTR ITSN1-S construct luciferase activity in HEK293 cells and 4.7-fold inhibiting in HeLa cells compared to the intact pTKluc vector. To identify putative regulation by microRNAs we plan to perform experiments using mutant constructs of 3'UTR of ITSN1-S. We also analyzed ARED database to find potential protein regulators of ITSN1 mRNA expression. It was shown that 3'UTR of ITSN1-S may contain a target site for TTP (tristetraproline) [Emmons et al., 2008]. We analyzed 3'UTR of ITSN1-L and identified the similar site for TTP. Our next step will be the analysis of interactions between TTP and mRNA of both forms of ITSN1, which could indicate a role of TTP in ITSN1 regulation. **Conclusions**. Using bioinformatical analysis and different experimental approaches for validation we have found that 3'UTR of ITSN1 contained multiple target-sites for different regulatory elements that may potentially affect ITSN1 expression in cells. To confirm these data additional experiments are needed.

Production and characterization of monoclonal antibody specific to Fibroblast Growth Factor Receptor 3 (FGFR3)

O. M. Gorbenko[1], G. V. Ovcharenko[1], N. O. Gaman[1], D. D. Volkova[1], D. Mayilo[1, 2], V. V. Filonenko[1], I. T. Gout[1, 3]

[1]Institute of Molecular Biology and Genetics NAS of Ukraine

[2]Taras Shevchenko National University of Kyiv

[3]University College London, London, UK

o.m.gorbenko@imbg.org.ua

Aim. To produce and characterize monoclonal antibody specific to Fibroblast Growth Factor Receptor 3 (FGFR3). **Methods**. PCR; Cloning, expression and purification of recombinant proteins; Hybridoma technology; ELISA; Western Blot; Immunoprecipitation. **Results**. The antigen for mouse immunization has been chosen by Bcepred software. S249C substitution was done by site-directed mutagenesis. The sequences corresponding to the loop II-III for FGFR3wt or FGFR3/S249C extracelular domain were amplified by PCR and cloned into pET42a. Recombinant proteins were expressed in BL21(DE3) cells as GST-His fused protein and purified by NiNTA affinity chromatography. GST-His/FGFR3/S249C-LII-III was used as antigen for mouse immunization and in primary hybridoma screening in ELISA and Western blot. Primary screening allowed us to select few positive clones, which recognized both GST-His/FGFR3wt-LII-III and GST-His/FGFR3/S249C-LII-III but not another member of FGFR family – GST-His-FGFR-1-LII-III that had been cloned and purified in our lab before. The selected positive clones were subcloned twice using limiting dilution method. Clones 37/2, 32/1, and 2/1 were successfully tested in WB and IP using insect cells infected with FGFR3wt and its mutant as well as in HEK293 cells transiently transfected by wt receptor and its mutant. **Conclusions**. The obtained positive clones 37/2, 32/1, and 2/1 were found to recognize both GST-His/FGFR3wt-LII-III and GST-His/FGFR3/S249C-LII-III and were successfully tested in WB and IP using insect cells infected with FGFR3wt and its mutant as well as in HEK293 cells transiently transfected by wt receptor and its mutant. Taken together we present in this work the characterization of three generated hybridoma targeting FGFR3, which could be a powerful tool for FGFR3 study.

Identification of novel FGFR1 tyrosine kinase inhibitors

A. A. Gryshchenko, V. G. Bdzhola, O. P. Kukharenko, S. M. Yarmoluk

Institute of Molecular Biology and Genetics NAS of Ukraine
andrey.grisch@gmail.com

FGFR1 relates to receptor tyrosine kinases which are important mediators of signal transduction in cells. FGFR1 has been detected in normal and malignant cells and is involved in biological events that include mitogenic and angiogenic activity; thus, it plays a crucial role in cell differentiation and development. It has been shown, that inappropriate expression or altered function of FGFR results in diverse pathologies, including tumorigenesis, obesity, rheumatoid arthritis and diabetic retinopathy, as well as vascular proliferative diseases such as atherosclerosis and restenosis. The aim of the present study is to identify novel FGFR1 inhibitors. FGFR1 kinase inhibition could provide new effective treatment of several diseases, including cancer. Receptor-based virtual screening technology was used for search for novel FGFR1 inhibitors. The docking of drug-like structures was performed with the program DOCK. Docked ligands were ranked accordingly to the energy score. The compounds with the best binding energies were evaluated visually and selected to biological tests. *In vitro* tests were carried out with -^{32}P-ATP assay. The molecular docking of 80000 compounds was performed. 210 substances were selected for *in vitro* testing. The biological tests revealed that 4 compounds had certain inhibition activity towards protein kinase FGFR1. IC_{50} of found inhibitors ranged in micromolar scope. For the compound 3-phenyl-benzo[c]isoxazole-5-carboxylic acid IC_{50} was 32 M and for N-{3-[5-(4-methoxy-phenyl)-thieno [2,3-d]pyrimidin-4-yloxy]-phenyl}-acetamide IC_{50} was 11 M. For two compounds 3-[2-(3-hydroxy-phenylamino)-thiazol-4-yl]-chromen-2-one and 5-Amino-1-(5-chloro-2-methyl- phenyl)-4-(6-methyl-1H-benzoimidazol-2-yl)-1,2-dihydro-pyrrol-3-one IC_{50} were of the same value 4.5 M. In this study new inhibitors of protein kinase FGFR1 were identified. These compounds represent 4 novel classes of FGFR1 kinase inhibitors. The identified classes will be used for further structural optimization to obtain more effective inhibitors of protein kinase FGFR1.

Single nucleotide polymorphism –131C G in the promoter region of chitinase-3 like 1 (*CHI3L1*) gene is not associated with glioblastoma risk and bad prognosis

A. V. Iershov, B. Boisselier[1], Y. Marie[1], S. El Hallani[1], M. Sanson[1], V. M. Kavsan

Institute of Molecular Biology and Genetics NAS Ukraine

[1]INSERM, U711, Biologie des Interactions Neurones & Glie,75013 Paris, France

a.yershov@yahoo.com

Aim. Chitinase-3 like 1 (*CHI3L1*, HC gp-39) gene belongs to glycosylhydrolase family 18 and, as we have found previously, is overexpressed in glioblastoma, the most aggressive type of human brain tumors. Understanding the reason of such changes in expression of *CHI3L1* gene could make it very useful as biomarker or target for tumor therapy. It has been shown that the level of *CHI3L1* gene expression in patients with asthma and schizophrenia correlates with SNP 131C G in *CHI3L1* promoter. It was important to find if the same reason leads to significant changes in *CHI3L1* expression level at the development of glial tumors and survival of patients with primary glioblastomas. **Methods**. Blood DNA from 296 glioblastoma patients without previous clinical history of glial tumor and 190 healthy volunteers was extracted using a standard saline procedure. The –131C G polymorphism was characterized by the Taqman SNP genotyping assay. –131C and –131G probes were labeled with FAM and HEX fluorescent dyes respectively at the 5'-end. Total RNA isolated by Qiagen reagent kit was used as a template for cDNA synthesis by random priming of reverse transcription (recombinant MMLV RT). *CHI3L1* gene expression was detected by SYBR Green real-time quantitative polymerase chain reaction (qPCR) using ABsolute QPCR SYBR Green ROX Mix. *ALAS1* gene has been chosen as an internal control. The Kaplan-Meier method and log-rank test were used for comparison of survival in different groups of glioblastoma patients with CC, GG, and CG variants in gene promoter. **Results**. qPCR confirmed the data found by SAGE and Northern analyses. Earlier it was shown that increased *CHI3L1* gene expression is associated with CC variant of CHI3L1 promoter in Hutterites asthma population and with GG variant in schizophrenia Chinese population. In contrast, our results obtained by Taqman SNP genotyping assay did not reveal the correlation between –131C G polymorphism either with *CHI3L1* expression level in glioblastoma or with survival of glioblastoma patients. **Conclusions**. Primary glioblastoma which appears *de novo* has different clinical and molecular features as compared with secondary glioblastoma, which arises from low-malignant gliomas. The *CHI3L1* gene expression is elevated in both primary and secondary glioblastomas and thus may be included along with several other genes into cDNA panel that can be used as a signature for this type of tumors. SNP 131C G in *CHI3L1* polymorphism in *CHI3L1* gene promoter leading to the increased expression of *CHI3L1* in asthma and schizophrenia is not associated with the expression level and bad prognosis for patients with glioblastoma. Apparently, other mechanisms participate in the elevation of the *CHI3L1* gene expression in this tumor.

Comparison of known 3D structures of human CYP 2E1, obtained by NMR, and a theoretical model of its spatial structure

V. O. Kitam

Institute of Molecular Biology and Genetics NAS of Ukraine
v.o.kitam@gmail.com

Cytochrome P450 2E1 (CYP 2E1) belongs to the monooxygenase cytochrome P450 depended system of hemthiolate enzymes and takes part in metabolism of xenobiotics. Noteworthy, that free radicals generated by CYP 2E1 result in intensification of lipid peroxidation, oxidative modification of proteins and nucleic acids, and development of the oxidative stress. The aim of this work was to compare a three-dimensional spatial model (3D structure) of human P450 2E1, previously built and optimized with the method of molecular dynamics (Danko I. M., Odynets K. O., Kitam V. O., Chaschin M. O., 2006), with experimentally defined structures of complexes of human CYP 2E1 with 4-methylpirazole (4-MP) and indazole (Porubsky et al., 2008). The quality of both theoretical and experimentally obtained models of spatial structures is high, however, side chains in the theoretical structure show less percent of unsolved amino acid rotamers and improper angles. Due to bigger distances between amino acids that form an active site of the protein (e. g. the distance between Phe207 and Phe298 is 7.62 Å in the theoretical structure, and 6.92 Å in experimentally defined structures; the distance between Leu210 and Leu215 is 5.65 Å and 4.89 Å, the distance between Leu103 and Leu110 is 8.90 Å and 6.61 Å respectively) the theoretical structure shows greater volume of an active center (2171 $Å^3$) in comparing with the experimentally obtained structures (878 $Å^3$ and 870 $Å^3$ for the complexes with 4-MP and indazole, respectively). A difference in the volumes of active center may explain mechanisms of inactivation of the enzyme activity by diminishing the volume of active center and its channel. Such construction of the channel makes it impossible for the substrates to penetrate into the active site. Consequently, the model calculated by us reflects a spatial structure of the active enzyme. It seems expedient to use the theoretical model of CYP 2E1, calculated by us, for subsequent research of mechanisms of various ligands placement within the active site and to predict possible inhibitors.

Morphogenesis of roots and shoots in *Gentiana pneumonanthe* L. tissue culture

I. I. Konvalyuk, N. B. Kravets[1], N. M. Strashniuk[1], V. M. Melnyk

Institute of Molecular Biology and Genetics NAS of Ukraine
[1]Ternopil V. Hnatiuk National Pedagogical University, Ukraine
konvalyuk@yandex.ru

Aim. *Gentiana* L. species provide an advantageous model to study the details of morphogenesis events. They are suitable for *in vitro* culturing to generate cell line-producers of valuable biologically active substances. Aim of the work was to specify conditions for organogenesis from the *G. pneumonanthe* L. calli obtained earlier. **Methods**. As a starting material were taken tissue cultures of root origin derived from the *G. pneumonanthe* two population plants (Koryukivs'ke forestry, Chernigiv region – at the 9th passage and v. Vygoda, Ivano-Frankivs'k region – at the 9th and 19th passages). To induce regeneration, we used MS medium as a basic one and supplemented it with combinations of phytohormones of diffrent concentrations: thidiazuron (TDZ) (1, 5, 10, 20 mg/l) and naphthylacetic acid (NAA) (0.01; 0.1; 0.2; 0.5; 1 mg/l). The nutrient medium MS with addition of 10 mg/l TDZ and 1 mg/l NAA was optimal for induction of the organogenesis. **Results**. Following two passages of the *G. pneumonanthe* calli culturing, the regeneration centers were formed upon illumination and at the end of the 3rd passage root and shoot were regenerated. Intensity of organogenesis depended also on the original plant genotype. In particular, comparison of the *G. pneumonanthe* calli of the same age (the 9th passage), but different in their origin, revealed that root number per explant (upon the same percent of rhizogenesis) in the tissue culture derived from Vygods'ka population plants was 21.7 root/expl., exceeding by 2.3 times this index (9.3 root/expl.) for the calli derived from other population plants (Koryukivs'ke forestry). The percentage of root and shoot regeneration in these two cultures was practically the same (17.4 % i 16.7 %, respectively), whereas shoot number per explant in the calli from Koryukivs'ka population plant was twice as much. The *G. pneumonanthe* callus capacity for organogenesis varied with growth duration. **Conclusions**. In particular, it was demonstrated that during cultivation of the *G. pneumonanthe* callus (v. Vygoda) from the 9th to 19th passage, the index of total regenerants number (roots and shoots) per explant decreased from 22 to 7 per/expl. This species showed rhizogenesis efficiency higher by one order of magnitude (in one case – even by two orders) than shoot organogenesis. Thus, *G. pneumonanthe* species exhibits high potential for shoot and root regeneration in tissue culture. An optimal for induction of organogenesis was nutrient medium MS, with addition of 10 mg/l TDZ and 1 mg/l NAA. The intensity of organogenesis depended on both genotype of original plant and duration of callus maintenance.

Determination of the mechanism of *FHIT* gene inactivation in clear cell renal carcinomas

A. G. Kondratov, S. M. Kvasha, V. V. Gordiuk, A. V. Rynditch

Institute of Molecular Biology and Genetics NAS of Ukraine
a.g.kondratov@gmail.com

Aim. *FHIT* is a well-known tumour suppressor gene which is located on human chromosome 3. It was shown that *FHIT* gene is frequently inactivated in different types of cancer. The main mechanisms of *FHIT* inactivation are mutations, deletions in DNA corresponding to the *FHIT* gene and methylation of its 5'-CpG-island. Since clear cell renal carcinomas frequently demonstrate lack of the FHIT protein expression without any genetic alterations we examined the methylation status of 5'-CpG-island of the *FHIT* gene in this tumour type. **Methods**. To determine methylation of 5'-CpG-island of FHIT, methyl-specific PCR (MSP) was used. Further, the results of MSP have been confirmed by bisulfite sequencing. Semi-quantitative PCR was used for determination of FHIT expression. Statistical analysis was done using the Student's test and the Fisher exact test with a two-tailed P. **Results**. The methylation status of *FHIT* gene in 22 paired clear cell renal carcinomas and normal renal tissues has been assessed. The hypermethylation of 5'-CpG-island of the *FHIT* gene has been detected in 12 (51.5 %) out of the 22 cases of clear cell renal carcinomas. The PCR products corresponding to methylated DNA have been found only in tumour samples but not in normal renal tissues. Bisulfite sequencing completely confirmed the results of MSP. Using semi-quantitative PCR we detected significant decreasing of the *FHIT* gene expression for the samples with detected methylation of its 5'-CpG-island and high level of expression in the samples with unmethylated 5'-CpG-island. It was revealed correlation of hypermethylation of 5'-CpG-island of *FHIT* gene with the patients age. It was shown that hypermethylation of *FHIT* gene is more frequently detected in patients over 50 years (10 out of 13 cases, 76.9 %) than in patients under 50 years (2 out of 9 cases, 22.2 %). **Conclusions**. For the first time the hypermethylation of 5'-CpG-island of the *FHIT* gene has been shown in clear cell renal carcinomas. Significant decreasing in *FHIT* gene expression has been revealed in tumours with hypermethylation of 5'-CpG-island of *FHIT* gene. Genetic abnormalities are rarely detected in *FHIT* gene in renal tumours, thus our finding concerning hypermethylation of *FHIT* 5'-CpG-island could be relevant for the explanation of inactivation mechanism for the *FHIT* gene in clear cell renal carcinomas.

Complex of translation elongation factors (eEF-1H): *in vitro* reconstitution from recombinant proteins

V. V. Lyudkovska, V. F. Shalak

Institute of Molecular Biology and Genetics NAS of Ukraine
vlada.lyud@mail.ru

Aim. Translation elongation step in higher eukaryotes requires the function of three different elongation factors, eEF-1A, eEF-1B and eEF-2. Eukaryotic elongation factor 1A (eEF-1A) is a 50 kDa G-protein that binds aminoacyl-tRNAs in a GTP-dependent manner and delivers them to A site of the ribosome. Regeneration of active eEF-1A*GTP form is assured by guanine nucleotide exchange factor eEF-1B, which contains three subunits , and . eEF-1B and eEF-1B catalyze the GDP/GTP exchange on eEF-1A, whereas the eEF-1B is considered to be a structural component. Complex of eEF-1A and three subunits of eEF-1B are called eEF-1H – the heavy form of eEF-1A. Although several models of macromolecular organization of this complex have been proposed, they are contradictory to each other with respect to subunit stoichiometric ratio and molecular masse of the complex. The aim of present study was to investigate eEF-1B complex formation from recombinant proteins using native gel electrophoresis and gel filtration approaches. **Methods**. The amount of eEF-1H complex in eukaryotic cells is too low to be sufficient for *in vitro* structural studies. That was the reason to create bacterial producent strains that allowed us to obtain preparative amounts of individual recombinant subunits. eEF-1B and eEF-1B were purified from *E. coli* as GST-fusion proteins with subsequent removing of GST moiety, whereas eEF-1B bearing polyhistidine tag was purified by affinity chromatography on Ni-NTA matrix. The activity of eEF- 1B and eEF-1B was checked by *in vitro* [^{3}H]GDP/GDP exchange assay. To visualize the complex of different recombinant subunits, we used agarose gel electrophoresis in native conditions. The molecular masses of reconstituted complexes was determined by gel filtration technique. After the gel filtration the protein composition of each peak was checked up by SDS PAGE. **Results.** By agarose gel in native conditions we readily detected stable eEF-1B , eEF-1B and eEF-1B complexes, when mixing equimolar amounts of each component. To determine molecular masses of the complexes, we performed gel filtration on the Superose 6 column. An analysis of individual subunits indicated unusual molecular masses of all of them, namely: eEF-1B was estimated to be 72 kDa, eEF-1B – 350 kDa and eEF-1B – 150 kDa. The theoretical molecular masses for these recombinant proteins were 27, 35 and 50 kDa, respectively. Gel filtration in the buffer of high ionic strength (0.5 M NaCl) containing chaotropic salt (0.5 M KSCN) did not change their elution profiles, that proves the absence of protein aggregates in the protein preparations. Mr of binary protein complexes eEF-1B , eEF-1B was determined to be 400 and 900 kDa, respectively. Stable ternary eEF-1B complex was also formed and according to SDS PAGE contained equimolar amounts of each subunit. Molecular masse of this complex was estimated to be more than 1 M Da. **Conclusions.** Individual recombinant subunits of elongation factor eEF-1B , eEF-1B and eEF-1B are able to form a stable high-molecular-masse complex *in vitro*. We consider that most probably eEF-1B and eEF-1B are not globular proteins, while eEF-1B may form a stable dimer. To define precisely the composition and molecular masse of eEF-1H complex the equilibrium sedimentation analysis is needed.

Cystathionine- -synthase expression in human placenta

O. P. Martsenyuk, B. Huppertz[1], M. Yu. Obolenskaya

Institute of Molecular Biology and Genetics NAS of Ukraine

[1]Department of Cell Biology, Histology and Embryology, Medical University of Graz, 8010 Graz, Austria

o.p.martsenyuk@imbg.org.ua

Aim. Human cystathionine- -synthase (CBS) is a unique pyridoxal phosphate dependent heme protein. It is a key enzyme in the *trans*-sulfuration pathway of homocysteine (Hcy). Its expression and catalytic activity are regulated at several levels. Clinical abnormalities in CBS deficiency are associated with many pregnancy complications. However, little is known about this enzyme in placenta. Our previous data have shown a positive correlation between Hcy and Cys content ($k_s = 0.7$, $p < 0.001$) and served as a basis to advance a hypothesis that *trans*-sulfuration pathway via cystathionine- -synthase (CBS) is active in human placenta. The goal of this study was to examine the expression and catalytic activity of CBS in human placenta. **Methods**. Placental samples were obtained from 3 term placentas (38–40 weeks) after noncomplicated pregnancies and from 3 first trimester placenta (8–10 weeks). The expression of CBS was examined by RT-PCR, Western blot and IHC analyses with corresponding antibodies. CBS enzyme activity was assessed by radiological method with [^{14}C]serine. **Results.** The expression of CBS in first trimester and term placenta was confirmed by RT-PCR and Western blot. It is mainly localized in trophoblast of villous chorion and endothelial cells of placental blood vessels. The CBS activity was evaluated in placental extracts with the addition of Hcy and [^{14}C]serine. The product [^{14}C]cystathionine was separated by column chromatography. The activity of CBS in term placenta was proven. It is known that human CBS is a redox sensitive heme protein. Toxic effects of Hcy have been attributed to direct or indirect perturbation of redox homeostasis. We suggest that the placental CBS catalytic activity may partly withstand the elevation of Hcy and maintain redox homeostasis via *trans*-sulfuration pathway. **Conclusions**. This is the first evidence to the presence of CBS enzyme in human placenta, which indicates that a functional *trans*-sulfuration pathway may be active in human placenta. The investigations were supported by grant of Ministry of Education and Science of Ukraine N 28-2008, INTAS YSF 06-1000014-5961.

A new tool for molecular ecology research

E. V. Moshynets, A. Koza[1], S. P. Shpylova, A. Spiers[1], V. A. Kordium

Institute of Molecular Biology and Genetics NAS Ukraine
[1]SIMBIOS, University Abertay Dundee, Dundee, Scotland, UK
moshynets@gmail.com

Soil is one of the most abundant of all ecological zones. Plant growth promoting rhizobacteria (PGPR) inhabit rhizosphere region as well as the surfaces of plant roots, and play an important role in plant health and crop productivity. Today our understanding of microbial community organization and functioning in situ is limited. Biological, chemical and physical interactions between members of the community and with plants occur over a wide range of physical scales, making the investigation technically challenging. Although the physical structure of the soil and root surfaces is recognized to play a significant role in the distribution of bacteria and the formation of local microbial assemblages (cenosis), direct investigation of the interaction between structure and community is hindered by an inability to recover samples without disturbing structure. Here we describe a plastic-film technology which is compatible with the modern microbial techniques such as fluorescent microscopy, FISH, DNA isolation and direct PCR amplification. In preliminary *in vitro* tests, we demonstrated bacterial attachment and detachment using the soil bacterium *Pseudomonas putida* KT2440, and the rhizosphere bacterium *P. fluorescens* SBW25 (both are regarded as PGPR). Bacterial attachment was found to be enhanced by pre-treating the films in soil water. Genomic DNA could be isolated from SBW25 attached to film samples using a genomic isolation kit, and direct PCR amplification of the 16S DNA sequence was possible using standard PCR conditions and Taq polymerase. Both flexible and more rigid plastic films have been tested in soil and rhizosphere microcosms, where fluorescent microscopy has been used to assess the interactions between GFP-tagged SBW25 and the native soil microbe community. Preliminary CT (Computerized Axial Tomography) scans suggest that the exact positioning of the film with regard to soil pores, aggregates and plant roots is possible, whilst SEM-EDX (scanning electron microscopy – Energy-Dispersive X-Ray analysis) experiments suggest that the two-dimensional chemistry of the soil-incubated film surface can be mapped at a scale relevant to microbial assemblages. This combination of two-dimensional sampling with modern molecular, chemical and structural techniques presents a powerful new tool for study on microbial communities in soil and rhizosphere.

ITSN1 and ITSN2 adaptor proteins function in common cell processes but differ in interactions with protein partners

O. V. Novokhatska, L. O. Tsyba, I. Ya. Skrypkina, O. V. Nikolaienko, O. V. Dergai, J. Moreau[1], A. V. Rynditch

Institute of Molecular Biology and Genetics NAS of Ukraine
[1]Institute Jacques Monod, Paris, France
olga.novokhatska@gmail.com

Aim. Intersectin (ITSN) is a multidomain adaptor protein that is conserved from nematode to human and functions as a scaffold for protein complexes assembly during clathrin-mediated endocytosis. In vertebrates ITSN family is represented by two genes that encode proteins with similar domain structure (EH1, EH2, SH3A-E, DH/PH and C2 domains). One of them, ITSN1, was extensively studied and shown to participate in endocytosis, actin cytoskeleton rearrangements, cell signalling and apoptosis while the function of another, ITSN2, was only recently linked to endocytosis process. In this work we intended to study ITSN2 interactions with protein partners, its possible specific role in endocytosis and involvement into other cellular processes. **Methods**. Molecular cloning, pull-down and immunoprecipitation, immunofluorescence study, microinjections into *Xenopus laevis* embryos. **Results and Conclusions**. During formation of clathrin-coated vesicle clathrin and GTPase dynamin mediate key stages of initiation and fission and considered to be endocytic marker molecules. Previously ITSN1 was shown to colocalize with these proteins and our results give the same picture for ITSN2 expression. To study ITSN2 function in comparison with ITSN1 we examined intracellular localization of both proteins. It is known that dynamin isoforms work at different cell compartments while colocalization of intersectins points that these proteins act in the same protein complexes likely in concert or competition. The possibility of oligomerization by coiled-coil regions (CCR) was also examined but neither binding ITSN1 CCR to ITSN2 nor immunoprecipitation of these proteins was detected. Furthermore ITSN2 interactions with protein partners were studied. Binding ITSN2 to dynamin 1 and Ras activator SOS1, that involves ITSN2 into mitogenic signaling, was mediated by the same interface (SH3A, C and E domains) as in case of ITSN1. Differences were observed in binding to ubiquitin ligase c-CBL, a new interacting partner Sema6A implicated in axon guidance and cytoskeleton rearrangements during development, and adaptor protein CIN85/Ruk, distinct SH3 domains of ITSN2 being involved in the binding to these proteins comparing to ITSN1. For better understanding of intersectins functioning we used *X. laevis* animal model for *in vivo* studies. ITSN2 functional domains were overexpressed in *Xenopus* embryos using microinjection technique. Overexpression of SH3A-E domains yielded into hyperpigmentation phenotype whereas DH/PH-C2 inhibited gastrulation stage. Previously, similar effect of hyperpigmentation was observed for CCR of Ral interacting protein (RLIP) that is responsible for interaction with endocytic adaptor Reps1, and was linked to actin cytoskeleton defects. Possible ITSN interaction with Reps1 was proved by *in vitro* and *in vivo* experiments, moreover genetic interaction of these proteins was shown in *C. elegans*. These data give the evidence for ITSN and Reps1 function in the same pathway likely affecting tightly connected processes of endocytosis and actin cytoskeleton rearrangements.

Phylogenetic study of the secondary structure of HIV-1 genomic RNA polyA hairpin

V. V. Otenko

Institute of Molecular Biology and Genetics NAS of Ukraine
rusco@voliacable.com

Aim. Due to peculiarities of HIV-1 retroviral replication polyA hairpins containing AAUAAA hexamer are located at the both ends of genomic RNA. To get a deeper insight into the mechanisms of polyadenylation suppression at the 5'-end and its stimulation at the 3'-end we carried out phylogenetical research of the polyA hairpin primary and secondary structures at the both ends of HIV-1 genomic RNA. **Methods**. For the analysis of the primary and secondary structures of the 5'- and 3'-ends of HIV-1 genomic RNA we used all corresponding nucleic sequences available in NCBI and LosAlamos databases. NC_001802 was taken as a Reference sequence. Secondary structure was predicted using mfold (Zuker) software. An additional software is also being developed to create custom databases of sequences and to perform analysis of their primary structure. **Results**. Primary and secondary structures of polyA hairpin were studied for a total of 352 isolates. 176 of them were taken for analysis of polyA region, identical sequences with the same Patient_id were excluded. It has been found that the most frequent base substitution set for the HIV-1 A, E-subtype is: A insertion between positions 9 and 10 (9A10), 39C39A and 44A 44G. All these base changes occur in hairpin stem and result in the appearance of two internal loops (AxA and UGxUG) instead of the second bulge in comparison with Reference sequence (NC_001802). For the C-subtype the most frequent base substitution is double mutation 38U39C 38C39U that results in the minor changes in the stem secondary structure. **Conclusions**. The phylogenetic analysis of the secondary structure of HIV-1 genomic RNA polyA hairpins showed the presence of subtype-specific base mutations, slightly altering the hairpin secondary structure, and the diversity of base mutations at the 5'- and 3'-ends of the genomic RNA.

Iinvestigation of mTOR-kinase isoforms

O. M. Skorokhod, I. O. Nemazanyy, G. G. Panasyuk, V. V. Filonenko, I. T. Gout

Institute of Molecular Biology and Genetics NAS of Ukraine
fiwinner@ukr.net

The mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase that regulates cell growth and metabolism in response to environmental signals affecting protein synthesis and cytoskeleton dynamics. The molecule of mTOR is a 290 kDa protein which consists of several functional domains: kinase domain; HEAT repeats that mediates protein-protein interaction; FRB domain interacts with the FKBP-rapamycin complex; FAT and FATC domains, which support catalytic activity of kinase domain. In mammalian cells mTOR could be involved in two major complexes: TORC1 and TORC2 in association with different proteins. Our previous studies have shown the possible existance of alternative mTOR isoforms. These isoforms could not only have different kinase activities but also may participate in different protein complexes and as a result play another role in signaling network compared to full-length mTOR protein. **Aim**. To find and prove the existence of mTOR isoform (TOR-) on RNA and protein levels in mammalian tissues and cell cultures. **Methods**. Molecular cloning techniques; RNA and DNA extraction; Northern blot; Western blot; reaction of immunoprecipitation; stable cell lines techniques. **Results**. In order to confirm the existence of mTOR isoforms the RT PCR on different cell cultures was conducted. Several forms with different cDNA length compared to the full-length mTOR form were found, cloned in bacterial vectors and sequenced. The bioinformatical analysis of mTOR-isoforms primary structure was carried out. It allowed to determine the absence of several functional domains among mTOR isoforms compared to the full-length molecule. Two of mTOR splice-variant clones, TOR51 and TOR50, were cloned into mammalian vector *pcDNA3.1* for stable cell lines generation, which will allow to make *in vitro* kinase reaction for investigation of TOR-kinase activity of these isoforms compared with native mTOR. A fragment of C-terminal part of mTOR was also cloned, overexpressed and purified from bacteria cells. This recombinant protein was used for TOR-specific polyclonal antibodies generation which were tested in Western blot and reaction of immunoprecipitation on panel of different cell lines and rat tissues. The results were compared with the commercially available (Cell Signaling) and previously obtained TOR-specific N-terminal antibodies. All of these antibodies detect not only major 290 kDa protein, but also several proteins with lower molecular weight, which could be mTOR isoforms. **Conclusions**. Several mTOR-isoforms were found with RT PCR technique and cloned. Bioinformatical analysis of mTOR-isoforms structure was performed. Two clones, TOR51 and TOR50, were used for stable cell lines generation. C-terminal fragment of mTOR was cloned, overexpressed, and a recombinant protein was used for TOR-C polyclonal antibodies generation. The future investigations for proving the existence of alternative forms of mTOR are necessary.

Cell-specific mechanisms responsible for regulation of the human Glutathione S-transferase P1 gene transcription

A. M. Slonchak, A. Chwieduk, J. Rzerzowska-Wolny, M. Yu. Obolenska

Institute of Molecular Biology and Genetics NAS of Ukraine
elephass@gmail.com

Aim. Glutathione S-transferase P1-1 is a widely distributed enzyme which plays a key role in protection of the cells from genotoxic damages. Although the regulation of GSTP1 gene expression is in the focus of numerous researchers, its cell-specific peculiarities are still poorly understood. The aim of the present finding was to clarify the mechanisms responsible for the different levels of GSTP1 expression observed in Me45, Hbl-100 and BeWo cells by assessing the CpG methylation of the promoter region and analyzing *cis*- and *trans*-acting factors implicated in the regulation of GSTP1 transcription. **Methods.** Hbl-100, Me45 and BeWo cells were cultured under the standard conditions. Level of GSTP1 mRNA was assessed by quantitative RT-PCR and level of GSTP1 protein by Western-blotting. Promoter methylation was analyzed by methylation-specific PCR (MSP). Truncated promoter fragments for the transient transfection assay were obtained by PCR and cloned into pGL3 basic. The Me45, Hbl-100 and BeWo cells were cotransfected with each of recombinant plasmids together with pRL-TK and luciferase activities were measured. Transcription factors interacting with GSTP1 promoter elements were assessed by competitive EMSA and supershift analysis. **Results.** Hbl-100, Me45 and BeWo cells exert different levels of GSTP1 expression. The highest level of GSTP1 mRNA and protein was detected in Hbl-100 and the lowest in BeWo. MSP revealed partial GSTP1 promoter methylation in Me45 and BeWo cells and no methylation in Hbl-100. Transient transfection assay provided the evidence for two positive (ARE and NF- B) and two negative (CRE and NF- B-like) regulatory elements similarly acting in GSTP1 promoter in three cell types. The results of competitive EMSA and supershift analysis indicated that ER together with unidentified protein binds to ARE and together with Fos binds to CRE sites in all cell types, but the structure of ER /Fos complex in Hbl-100 differs from that in Me45 and BeWo. In addition, the NF- B interaction with NF- B site was identified as a p50/p50 homodimer in BeWo and p50/p65 heterodimer in Hbl-100 and Me45 cells. **Conclusions.** Thus, we analyzed for the first time the molecular mechanisms responsible for the steady-state level of GSTP1 transcription in Hbl-100, Me45 and BeWo cells. The obtained results clearly indicate that promoter methylation and transcription factors are responsible for the cell-specific levels of GSTP1 expression in these cells. We assume that CpG methylation accounts for the lower GSTP1 expression level in Me45 and BeWo comparing to Hbl-100 cells, while further down-regulation observed in BeWo results from the absence of NF- B p65 transactivating subunit in this cell type. We also demonstrated for the first time that the interaction between ER and other transcription factors occurs in the regulation of GSTP1 transcription.

Search for inhibitors of human protein kinase ASK1

O. V. Sovetova, O. P. Kukharenko, V. G. Bdzhola, G. P. Volynets, S. M. Yarmoluk

Institute of Molecular Biology and Genetics NAS of Ukraine
sovetova@bigmir.net

Apoptosis signal regulating kinase 1 (ASK1) plays an essential role in stress and immune response and can be related to the development of several diseases. It has been proven by genetic experiments, that blocking ASK1 damps apoptosis, induced by cytokins TNF , Fas, or by hypoxia, and also stops the reactions of inflammation caused by different factors in cells. Inhibitor of ASK1 could become highly-effective therapeutic agent in treating many acute pathological states of different diseases. Despite the active search for specific inhibitors of Ask1 by many pharmaceutical companies and research institutes, effective compounds have not been found until now. Therefore, the aim of this work is search and design of ASK1 inhibitors. The search is based on the following methods: receptor-based virtual screening of small organic compounds library; biochemical estimation of the biological activity of compounds by *in vitro* kinase reactions (-^{32}P-ATP method). Initially, the docking of the library of 75000 organic compounds in the ATP binding pocket of ASK1 was performed. As a result of the virtual screening, over 200 compounds were selected for the biological tests by *in vitro* kinase assay using commercial enzyme-catalytic domain of ASK1. Several compounds revealed the activity with IC_{50} from 1 to 10 M. A new class of ASK1 inhibitors 2-thioxo-1,3-thiazolidin-4-one has been found. The values of IC_{50} were defined strictly for five compounds from this class. The most active compounds have IC_{50} 2.5 M. Thus, the obtained results are reasonable to continue the search for more active compounds among the class of 2-thioxo-thiazolidin-4-one by rational design with directed synthesis of new structures. The other, less active chemical classes, found at the screening, are also revised and considered as promising structure.

Determination of copy number in *SMN1* gene using Real-Time PCR

O. O. Soloviov, L. A. Livshits

Institute of Molecular Biology and Genetics NAS of Ukraine
fancyflight@yandex.ru

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive diseases that leads to the anterior horn cells damage. SMA has a prevalence of 1/6000 to 1/10000 newborns and a carrier frequency of 1/40 to 1/50. It is caused by homozygous deletion of the survival motor neuron gene 1 (SMN1). The highly homologous gene *SMN2* is present in all patients but cannot compensate for loss of *SMN1*. *SMN2* differs from *SMN1* by a 5 nucleotide changes but a C T transition in exon 7 leads to the exon skipping. Approximately 94 % of SMA patients lack both copies of *SMN1* exon 7, which can be easily detected by RFLP. Using this analysis the presence or the absence of *SMN1* gene can be determined but it is not possible to estimate correctly *SMN1* copy number because of partial digestion and lack of quantitative assessment. For these purposes we have developed an easy and reliable quantitative Real-Time PCR method using SYBR Green. We have chosen ALB gene (albumin) as a reference. The primers for *SMN1* gene amplification were designed to achieve allele specificity between *SMN1* and *SMN2*. *SMN1* and ALB Real-Time PCR assays were optimized so that the amplification efficiencies of these genes were near 100 % and within 5 % of each other for Livak calculation method evaluation. To validate the quantitative analysis of *SMN1* gene copy number we have taken as control samples four SMA patients and their 8 parents, which have been previously tested using standard RFLP method. The patients with homozygous deletion of *SMN1* showed absence of amplification, the carriers with one *SMN1* copy showed the increase of C_t value (C_t = = 0.49 1.09) compared with that of albumin. The C_t ratio means in normal controls (two copies of *SMN1* gene) were 0.96 1.028 and in carriers of one *SMN1* copy – 0.406 0.616. No overlap was observed between *SMN1* heterozygous deletion carriers and normal controls. So, the quantitative analysis of *SMN1* copy number is a fast and sensitive method that can be applied for carrier analysis of SMA in Ukraine.

A design of protein kinase CK2 inhibitors based on the 2-oxy-1,2-dihydroquinolin-3-il acetamides derivatives

A. R. Synyugin, M. A. Chekanov, S. S. Lukashov, S. M. Yarmoluk

Institute of Molecular Biology and Genetics NAS of Ukraine
drumms@mail.ru

Recently, the search for new protein kinase inhibitors as a potential antiviral and anticarcinogenic drugs is in the focuse of researchers. Since casein kinase 2 (CK2) has enhanced activity in a wide spectrum of tumours, its usage by many viruses for phosphorylating of own proteins, and inhibition of apoptosis cellular mechanisms, CK2 kinase can be confidently considered as a target for creation of new drugs with directed action. As a result of computer design by the method of flexible doking (programm package DOCK 4.0), 30 compounds, having the highest affinity to the surface of ATP-binding site of the enzyme, were selected from a virtual library of about 3000 derivatives of quinoline-2-one. A few inhibitors of CK2 kinase with IC_{50} of 16–50 M were found in the series of synthesized compounds using *in vitro* testing with -marked ATP. The predicted inhibiting activity was found in the series of 2-(7-metoxy-2-oxy-1,2-dihydroquinolin-3-il)-N-arylacetamides. The obtained data specify the direction of subsequent chemical optimization of the inhibitor structure, which will be conducted in further work.

Generation and characterization of single-chain antibody and fluorescent protein fusions

M. V. Tsapenko, A. I. Flyak[1], O. B. Gorbatiuk[1], I. M. Gilchuk[2], P. V. Gilchuk[2]

Institute of Molecular Biology and Genetics NAS of Ukraine

[1]Kyiv Taras Shevchenko National University, Ukraine

[2]Institute of Genetic and Regenerative Medicine of the AMS Ukraine

mtsapenko@ukr.net

Monoclonal antibodies conjugated with fluorescent tags are valuable as highly-specific molecular probes for different immunoassays and cell separation procedures. The labeling of antibodies is traditionally accomplished by their chemical coupling with different organic fluorophores that requires relatively large quantities of purified antibodies, a chemically activated tag, and must be held under controlled conditions of the reaction. Complexity of the process of hybridoma generation and monoclonal antibodies production, and necessity of chemical coupling stage, altogether, result in high cost of the obtained probes. Advanced phage antibody and gene manipulation technologies facilitate recombinant antibodies production and allow their genetic fusion with other protein partners. Such recombinant proteins can be produced in bacteria by rapid and cost-effective fermentation process under defined conditions. Thus, the recombinant antibodies genetically fused with fluorescent proteins are an attractive alternative to their chemical conjugates with fluorescent dyes. The aim of this work was to accomplish the genetic fusion of anti-human interferon- 2b single-chain antibody (scFv) with fluorescent proteins (FP) and to investigate their applicability for fluorescence based immunoassays. The following methods and techniques were used in this work: bacteria cells culturing, gene cloning, polymerase chain reaction, DNA electrophoresis, DNA sequencing, ELISA, Western-blotting, protein expression, protein purification, and protein refolding, fluorescent microscopy. The genes of fusion proteins that consist of ScFv and FP moieties were designed and expressed in *E. coli*. EGFP and mCherry, which emit, respectively, in green (508 nm) and red (610 nm) spectra and have high brightness and photostability, were selected as fusion partners for ScFv and FP. The expression yield of the target proteins was optimized and amounted up to 500 mg scFv-FP from 1 l of *E. coli* culture. The fusion proteins have been obtained on multi-milligram scales in purified and soluble form with rapid and cost-effective on-column refolding process. Immunochemical and immunofluorescent assays have confirmed that both moieties of scFv-FP fusion proteins maintain their functional activity after the refolding. Using antigen coated agarose beads, the applicability of ScFv-FP for fluorescence-based immunoassays has been shown.

Novel aryl and heteryl amides of 9-substituted phenazine-1-carboxylic acid and their biological activity

O. V. Vasylchenko

Institute of Molecular Biology and Genetics NAS of Ukraine
rediskin@ukr.net

Within the last 50 years, several phenazine derivatives showing antimicrobial activity against fungi, yeasts and bacteria have been discovered. Natural phenazines are produced by *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium* and *Burkholderia*. At present, approximately 50 natural and synthetic phenazine compounds differing only in modifications of the parent heterocycle have been described. Generation of structural modifications mainly leads to phenazines with different physical properties and changes in the spectrum of their activity. Although the molecular mechanisms of phenazine derivatives action are as yet poorly understood, the antibacterial activity may be explained by inhibition of cellular superoxide dismutase and, or by inhibition of bacterial DNA-dependent RNA polymerases. The aim of this work was the synthesis of two new series of arylamides of 9-substituted PCA, because such modification is known to influence considerably the activity of the tricyclic geteroaromatic carboxylic acids. It is also well known that RNA synthesizing machinery is a key cellular target for several drugs against bacterial pathogens. Taking into account the topological and functional similarity of polymerases, and the fact that the T7 RNA polymerase does not require a radioactive technique to evaluate and visualize transcription products, we used it as an *in vitro* model to study polymerase inhibition by the set of PCA derivatives. Following the convenient methodology developed by us, we prepared an extensive set of 9-substituted PCA-1-carboxamides whose amide fragments were formed by the alkyl groups or aryl-, heteryl-ring systems bearing exocyclic groups at different ring positions. The new synthesised compounds exhibited significant, structure-dependent activity in cell-free RNA transcription system. We investigated the *in vitro* activity of new N-aryl and N-heteryl 9-substituted phenazine-1-carboxamides against different bacterial strains. The results obtained show a moderate activity of these compounds against Gram-positive and Gram-negative bacteria. The molecules selected will be modified further to improve their biological activity and to investigate the molecular mechanism of their action. **Acknowledgment.** The author offers sincere thanks to V. G. Kostina, V. V. Negrutska, O. M. Deriabin for assistance.

Generation of HEK293 stable cell lines overexpressing recombinant human PTEN and its inactive mutant PTEN/C124S

D. D. Volkova[1], V. S. Gryshkova[1], V. V. Filonenko[1], I. T. Gout[1, 2], O. M. Gorbenko[1]

[1]Institute of Molecular Biology and Genetics NAS of Ukraine

[2]University College London, London, UK

drvlkv@gmail.com

Aim. PTEN is a tumor suppressor gene mutated in many human sporadic cancers and in hereditary cancer syndromes. PTEN protein is a phosphoinositide-3-phosphatase that acts in direct antagonism to growth factor stimulated PI3-kinases by metabolizing phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3). A wealth of data has now illuminated the pathways that can be controlled by PTEN through PtdIns(3,4,5)P3, some of which, when deregulated, give a selective advantage to tumour cells. There are data about implication of PTEN in both PI3K-dependent and -independent way in such crucial processes as promotion of cell cycle arrest, apoptosis, inhibition of cell cycle motility, insulin signaling and adipogenesis. Because of its clinical importance, PTEN is now a subject of intense study in many laboratories. Recently we have identified adypocyte lipid binding protein FABP4 as a PTEN binding partner. To investigate the role of phosphatase activity of PTEN in this interaction we have generated HEK293 cell lines stably expressing recombinant human PTENwt and its phosphatase dead mutant PTEN/C124S. **Methods.** To generate and analyze these model lines we applied a standard technique. cDNAs corresponding to PTENwt and PTEN/C124S were cloned into eukaryotic expression plasmid *pcDNA3.1*(+). The generated constructs *pcDNA3.1*/PTENwt and *pcDNA3.1*/PTEN/C124S were linearized by *MefI* emzyme and transfected into HEK293 cell line. The cells were selected on G418 for 4 weeks and then subcloned using disc cloning procedure. PTEN expression in obtained stable cell lines was checked by WB with anti-PTEN N19 antibody and was quantitatively higher than in control HEK293*/pcDNA3.1* cells. This control line was generated in our lab earlier. As a loading control, the antibodies to -actin were used. **Results.** The generated HEK293/PTENwt cell line was used in investigation of PTEN-FABP4 interaction in mammalian cells. GFP-FABP4 construct was transiently transfected into each cell line and after cell treatment with 1 mM H_2O_2 PTEN-FABP4 complex formation was detected by co-immunoprecipitation with anti-GFP antibody followed by WB with anti-PTEN N19 or anti-FABP4 H81 antibodies. As a negative control, transient transfection by GFP alone was used. **Conclusions.** Taken together we have generated two human embryonic kidney cell lines stably expressing either recombinant human PTEN or its inactive mutant PTEN/C124S, which could be a powerful tool for PTEN study. Using these lines we have already shown that PTEN-FABP4 complex could be detected in mammalian cells in the presence of H_2O_2, suggesting that PTEN activity plays no role in this interaction.

Study of biological activity of low-molecular thymic factors on CBA line mice lymphocytes

U. O. Yakubovsky, L. A. Zaika, O. I. Bolsunova, A. I. Potopalsky, V. V. Filonenko

Institute of Molecular Biology and Genetics NAS of Ukraine
jun@email.su

Elaboration of new immunomodulating preparations is an important problem, especially preparations from thymus, modification of which considerably extends their application in medicine. Based on this position we determined as the main purpose of this research the study of the influence of thymus hormones on stimulation of lymphocytes proliferation. At this stage the acquisition of extracts from thymus and selection of their lymphocytopoiesis stimulating matters (LSM) were conducted. The influence of isolated thymic factions on blast-cell transformation ability of lymphocytes (RBTL) was simultaneously studied. This approach enabled us to check up the activity of separate components of total preparation as to blast-cell transformation activity of lymphocytes, so that at the next stage it would be possible to determine the nature of biologically active substance. During this research the influence of thymic extract, LSM and some separated low-molecular fractions on proliferative activity of lymphocytes of CBA line mice under different doses (100–0.0001 g/ml) was studied. These experiments show that thymus extract for certain stimulated proliferation of splenocytes only in a dose of 100 g/ml (256 16.1 – experiment; 101 1.9 control IS (index stimulation) = 2.5. LSM was more active than a total thymus extract and in a dose of 100 g/ml enhanced IS of RBTL up to 3.6. This preparation in various concentrations (from 10 till 0.0001 g/ml) also showed more pronounced effect than the extract and control. Analyzing LSM fractions it is safe to state, that they stimulate proliferation of splenocytes in considerably lower doses, than initial total preparation. The second fraction was considerably more active than the control only at doses of 0.01–0.0001 g/ml. The third fraction activated essentially lymphoid cells proliferation at doses of 1–0.001 g/ml) as against the control. Noteworthy, that the third fraction shows its activity in a wider range of concentrations, considerably stimulating lymphocytes in comparison with the control. It has been shown that influence of the preparations in combination with such mitogens as PHA, ConA and LPS on blast-cell transformation of lymphocytes more or less stimulated RBTL in all explored doses for certain. The results presented suggest that these investigations should be continued.

A role of isatizone in plant defense against necrotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000

I. E. Zaets, N. V. Chervatyuk

Institute of Molecular Biology and Genetics NAS of Ukraine
zkora@ukr.net

Plants defend themselves against pathogens, herbivores and abiotic stressors by mounting a variety of biochemical and physiological defenses which results in constitutive and inducible resistance (systemic acquired resistance, induced systemic resistance). A set of chemical compounds induces systemic resistance in plants against pathogens and predators. Izatizone is an antiviral commercial preparation where methysazone exerts inhibitory effect on the virus propagation. **Aim.** In this study some molecular and biochemical mechanisms of Izatizone action on the growth and protection of *Arabidopsis thaliana* (L.) Heynh against necrotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) were examined. **Methods**. Plant RNA isolation, cDNA synthesis, real-time PCR with PR2, LOX2 primers. **Results**. Izatizone was not toxic for *Escherichia coli* and *Pst* in dilution of commercial formulation 1:20, and this concentration exhibited protective capacity against *Pst* DC3000. A plant growth promotion was not detected after application of series of preparation dilutions. Activities of key antioxidant enzymes (guaiacol peroxidase, glutathione-S-transferase) associated with the plant defense system were different in Izatizone treated and control plants (non-treated). Increase of enzyme activities after plant treatment with the preparation and decrease after infection by pathogen in contrast to the control, may be one of the crucial mechanisms in the plant defense system against necrotrophic pathogens. The results of qRT-PCR exhibited that plant treatment with Izatizone did not initiate *de novo* transcription of plant defense gene PR2, encoding -1,3-glucanase, and so far did not induce systemic resistance on SAR-type. However, elevated expression of the LOX-gene, encoding lipoxygenase, indicated putative defense mechanism formed via Ja-dependent signal transduction pathway. **Conclusion**. Further researches will be concentrated on studying Izatizone influence on the putative Ja-dependent signal transduction pathway.