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Collection of Theses

Identification of a novel CoA synthase isoform and its interaction with SH3 domains of signaling proteins *in vitro*

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CoA synthase is a bifunctional enzyme which mediates the final stages of CoenzymeA biosynthesis. CoA and its derivatives are important players both in cellular metabolism and signal transduction. Recently we discovered, cloned and characterized a new splice isoform of CoA synthase named CoASy β and originally identified CoA synthase CoASy α. The transcript specific for CoASy β was identified by electronic screening and by RT-PCR analysis of various rat tissues. The existence of this novel isoform was further confirmed by immunoblot analysis with antibodies directed to the N-terminal peptide of CoASy β. Using confocal microscopy, we demonstrated that both isoforms are localized in mitochondria. The N-terminal extension does not affect the activity of CoA synthase, but possesses a proline-rich sequence which can bring the enzyme into complexes with signaling proteins containing SH3 or WW domains. Therefore, we have supposed that 29 aa extension may function as an adapter sequence to form regulatory complex of CoASy β with signaling apparatus of the cell. To test this suggestion and to identify novel binding partners that specifically interact with CoASy β N-terminal extension through their SH3 domains we have performed in vitro pull down experiments. We cloned CoASy β 29 aa extension as a fusion with His- and GST- tags in prokaryotic expression vector pET 42a, expressed in E. coli BL21 cells and purified it on NiNTA agarose. A panel of bacterially expressed SH3 domains from various proteins was previously created and kindly provided to us by Prof. I. T. Gout. Hence, we immobilized His fused CoASy β N-terminal peptide on NiNTA agarose and performed pull down with a set of SH3 domains. In general, binding SH3 domains of 13 proteins was tested and for 5 of them specific interaction with CoASy χ N-terminal extension was confirmed. Thus, 29 aa sequence can indeed mediate interaction with SH3 domains of signaling proteins and CoASy β may be involved in signaling complexes formation in living cells.

Novel isoform of intersectin 1 with new 3' end

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The Intersectin 1 (ITSN1) protein functions both in clathrin-mediated endocytosis and in MAP kinase signaling. Intersectin (ITSN1) is a multidomain cytosolic membrane-associated adaptor protein that coordinates indirectly with endocytic membrane traffic with the actin assembly machinery. ITSN1 is a binding partner for dynamin, Cdc42, Sos1, WASL, Eps15 and potential partner for protein disrupted in schizophrenia (DISC1). The complex domain structure comprises two EH, CCR and five SH3 domains in the short isoform, plus RhoGEF, pleckstrin, and putative calcium-interaction domains in the long isoform. Many additional splicing events, affecting different parts of ITSN1, were demonstrated in our laboratory. We identified a new ubiquitously expressed isoform of ITSN1, which possesses novel C-terminus. This transcript encodes protein consisting of two EH domains, coiled-coil region, SH3A domain and novel 116 amino acid residues encoded by exon 22a. We identified two additional splicing events affecting this isoform, namely, splicing of exon 6/6' and brainspecific splicing of exon 20. It is evidence of the existence of four transcriptional isoforms containing exon 22a. C-terminus of novel isoform contained predicted transmembrane segment and potential binding motifs for amphiphysin, PI3K regulatory subunit and SH3A domain of ITSN1. We suppose that the novel ITSN1 isoform could play a role of a negative regulator of endocytosis, as truncated proteins of ITSN1 possess such effect on clathrin-mediated endocytosis (Tong-Wey Koh et al.). Subcellular localization of novel ITSN1-22a isoform and fragment encoded by exon 22a was determined. Truncated protein, lacking SH3 domains, is known to translocate to the nucleus. Two nuclear localization signals were predicted in the CCR. We demonstrated that interactions via SH3A domain are sufficient to maintain cytosol localization. Peptide encoded by 22a exon spreads through the cytoplasm uniformly. We found that exon 22a is represented by a part of mobile element LINE2c. Analysis of genomic sequences revealed absence of such exon in Xenopus laevis and Danio rerio. We suppose appearance of this alternative exon in mammals as LINE elements are peculiar to mammals and absent in other groups.The study was supported by INTAS (project no.05-1000004-7762).

Binding specificity of SH3 domains can be regulated by alternative splicing

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SH3 (src homology 3) domains are small protein modules containing approximately 50 amino acid residues. They are found in a great variety of intracellular or membrane-associated proteins including proteins with enzymatic activity, adaptor proteins, and cytoskeletal proteins. The SH3 domain has a characteristic fold which consists of five or six beta-strands arranged as two tightly packed anti-parallel beta sheets. The region bound by the SH3 domain is proline-rich in all cases and contains PXXP as a core-conserved binding motif. Intersectin1 (Itsn1) is a cytoplasmic membrane-associated protein that coordinates indirectly endocytic membrane traffic with the actin assembly machinery. In addition, the encoded protein may regulate the formation of clathrin-coated vesicles and could be involved in synaptic vesicle recycling. This protein has been shown to interact with dynamin, Cdc42, SNAP23, SNAP25, N-WASP, Sos1, CBL, EPS15, etc. Multiple transcript variants encoding different isoforms have been found for this gene. Itsn1 short isoform consists of two EH domains at its N-terminus, five tandem arranged SH3 domains. Long isoform has additional three domains (DH, PH, and C2) as a C-terminus extension. Previously alternative brain-specific exon affecting SH3A domain was found in our laboratory. This exon encodes five additional amino acids in the n-Src loop of SH3A domain and potentially is capable of affecting the binding of SH3A domain to certain partners. To study binding specificity of Itsn1 SH3A domain to some proteins we have obtained two His-tagged alternative isoforms of SH3A and GST-fusion proline-rich domains of c-Cbl, Cbl-b, Sos1. We failed to obtain N-WASP proline-rich domain (PRD) using bacterial expression system, so its ORF was cloned in pcDNA4MAX vector and expressed in COS7 cell line. Flag-tagged Ruk was a generous gift of Dr. L. B. Drobot.To explore PRD/SH3A interaction a series of pull down experiments were undertaken. Brain-specific isoform of SH3A domain had slightly higher affinity to Ruk and decreased one towards Cbl-b, c-Cbl and mSos1. Affinity of Itsn1 SH3A domain to N-WASP was not significantly affected by mentioned 5 amino acid insertion, which potentially evoke rearrangement of charged groups, engaged in interaction interface. Analyzing sequences of SH3 domains we propose a model in which insertion of 5 amino acids moves the pair of negatively charged side chains to interface influencing on interactions with PRD containing charged side chains. Hence, binding affinity of SH3 domains to partners can be regulated by introducing small alternative exons in tissue and developing specific manner. *The study was supported by INTAS (project no.05-1000004-7762).*

Generation and characterization of monoclonal antibodies against phosphate cotransporter NaPiIlb

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Ovarian cancer is the most common gynecologic malignancy that usually becomes far advanced before it is diagnosed. Therefore, it is associated with poor prognosis and survival rate. So far, only a few tumor-associated markers and antigens, specific for ovarian cancer, have been identified. One of them, MX35 antigen, was originally found nearly 20 years ago at Memorial Sloan-Kettering Cancer Center as unknown antigen through the use of monoclonal antibodies termed MX35 (NewYork, USA). MX35 mAb was obtained from mice immunized with fresh ovarian carcinoma cells and selected by extensive analysis of normal and malignant tissues and cell lines. mAb recognizes MX35 antigen which is overexpressed in 90 % ovarian cancer specimens, some ovarian cancer cell lines, including OVCAR3, and shows restricted expression in normal tissues. MX35 antigen has recently been identified as a sodium-dependent phosphate transporter NaPiIIb (SLC34A2, NaPi3b, Npt2) in our laboratory by screening OVCAR3 cDNA expression library with MX35 mAb (IMBG, Kyiv, Ukraine). Later on, NaPiIIb was also confirmed as MX35 antigen by affinity purification followed by mass spectrometry (New York, USA). NaPiIIb belongs to SLC34 family of sodium-dependent phosphate transporters and is involved in regulation of inorganic phosphate metabolism and the maintenance of phosphate homeostasis via inorganic phosphate absorption from the small intestine. The data concerning expression of NaPiIIb in different tumor tissues are very limited. The aim of our work was to generate monoclonal antibodies against a large extracellular loop (ECL) of NaPiIIb for further investigation of NaPiIIb as a potential tumor marker. Initially we proposed the model of NaPiIIb with 8 transmembrane domains, ECL (188–366 aa) and cytoplasmic N- and C-tails due to similarity to highly homologues phosphate transporter NaPiIIa. The fragment of NaPiIIb gene, corresponding to ECL (188−361aa), has been cloned into bacterial expression system. Recombinant protein was expressed, purified and used for mice immunization. Generated hybrid clones have been selected in ELISA and Western-blot analysis by recombinant NaPiIIb/ECL. The ability of generated antibodies to recognize the native NaPiIIb protein was confirmed in Western-blot analysis and immunohistochemistry on ovarian cancer tissues and cell lines. Thus, we have generated monoclonal antibodies against NaPiIIb, recognizing native protein, and will use them for further investigation of phosphate cotransporter NaPiIIb.

Channeling of tRNA during initiation and elongation steps of translation

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*The elongation of polypeptide chain requires efficient delivery of aa-tRNA to the ribosome, and the delivery of deacylated tRNA back to AARS for re-charging with amino acid. In higher eukaryotic cells molecules of tRNA are always bound to some macromolecules, such as AARS, ribosome or elongation factor eEF1A and are transferred from one macromolecular complex to another in a non-diffusional way. This process is named «tRNA channeling». Recently the possibility of eEF1A in GDP bound state was revealed to form a stable ternary complex with deacylated tRNAs. The aim of our research was to find out the basis of such ternary complex formation. Here we show the formation of ternary complexes of eEF1A1*GDP with tRNA specific for Phe, Tyr, Ser, Met, Ile, Leu, Pro, and Asp. Dissociation constants for all these complexes measured by band retardation assay comprised 250, 750, 350, 600, 470, 600, 200, 750 nM, respectively. K_d values for the formation of similar complexes with eEF1A2 – tissue specific isoform of elongation factor 1A – were found to be 250, 400, 120, 250, 100, 150, 50, 250 nM, respectively. Thus, eEF1A2*GDP binds most of the tRNA much stronger than eEF1A1*GDP. Also, we demonstrated the formation of the ternary complex of initiation factor 2 (eIF2) in GDP bound state with deacylated initiator $tRNA_i^{Met}$ (K_d is ~160 nM). Moreover, we found that elongator $tRNA_e^{Met}$ was also capable of forming a complex with eIF2*GDP (K_d ~100 nM). Other elongator tRNAs were not capable of interacting with eIF2*GDP. The initiator $tRNA_i^{Met}$ was also studied for the ability to form a ternary complex with eEF1A1*GDP or eEF1A2*GDP. Surprisingly, $tRNA_i^{Met}$ was found to be capable of forming both ternary complexes, with the affinity comparable to elongator tRNAs. It is known that $tRNA_i^{Met}$ from yeast and plants, but not from mammals, has antideterminants for eEF1A in position 64 preventing interaction with the elongation factor. The importance of position 64 in tRNA structure for the interaction with eEF1A*GDP was examined. Three mutant $tRNA_3^{Lys}$ containing inserts of the wobble G:U base pairs instead of the usual base pairs in T-stem were prepared using T7-polymerase reaction. For all mutant and control tRNAs the K_ds of the ternary complexes with both isoforms of eEF1A were measured. No effect of the mutation on the ternary complexes formation was detected. Only in case of eEF1A2 some increase of K_d for the mutant tRNAs was found if the mutation was made in position 64:50. The data suggest that formation of non-canonical complexes with eEF1A*GDP is possible with any mammalian tRNA, including initiator $tRNA_i^{Met}$. Importantly, eIF2*GDP was capable of interacting with both initiator and elongator $tRNA^{Met}$. On the basis of our data we conclude that channeling mechanism can work both at the initiation and elongation steps of mammalian protein synthesis.*

Alternative splicing of intersectin 1: expression analysis and cloning of full-length human ITSN1 transcripts

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Alternative splicing has recently become one of the major mechanisms, increasing the diversity of transcriptom and found important application both in physiology and the study on development and genesis of diseases. Intersectin 1 (ITSN1) is an evolutionary conserved, multidomain protein involved in clathrin-associated endocytosis, signal transduction, and cytoskeleton rearragement. ITSN1 mRNAs encode two major isoforms, both short and long. The short form is ubiquitously expressed and consists of two N-terminal EH domains, a coiled-coil region, and five SH3(A-E) domains. The long form is predominantly expressed in neurons and includes three additional C-terminal domains, namely, DH, PH, and C2. The aim of this work was to analyze the expression of different ITSN1 isoforms in human tissues and to clone full-length human ITSN1 transcripts comprising different combinations of alternatively spliced exons. In this work we used methods of molecular cloning and semiquantitative PCR. Recently, we have identified eleven alternative splicing events affecting mouse and human ITSN1 transcripts. Five of them introduce premature translation termination codons and, possibly, lead to mRNA degradation. Other isoforms have deletions or insertions in resulting protein, which may affect binding properties and functions of ITSN1. Here we present the results on cloning and characterization of fifteen full-length human ITSN1 transcripts comprising different combinations of alternatively spliced exons. Moreover, we have found additional alternative splicing event affecting exon 5 of human ITSN1 gene. Expression analysis of long ITSN1 isoform and isoforms with skipping of exon 20 and 35 revealed lower level of expression in human glial tumors. Short isoform and transcripts with skipping of exons 25−26 and insertion of exon 22a did not change expression in these tumors. Analysis of GenBank databases showed the possibility of existence of exons 1a and 1b (EST DA507184 and DA738410), which potentially can regulate translation of ITSN1. Their expression was found non-uniform in different human normal and cancer tissues. Thus, alternative splicing creates a variety of transcriptional and protein ITSN1 isoforms with potentially different functions in regulation of endocytosis and signal transduction.

Heterological expression of mice cytochrome P450 2E1 gene in *Escherichia coli*

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Cytochrome P450 2E1 (Cyp2e1) is a monooxigenase from cytochrome P450 family, which participates in oxidative metabolism of more than 80 endogenic and exogenic low molecular weight hydrophobic substances, particularly drugs, converting them to water soluble substances, easily rejected from the body. On the other hand, the important function of Cyp2e1 is a transformation of procancerogenes and protoxins into reactive metabolites which cause the damage of the cells. The aim of the study was to obtain and characterize the protein producer of recombinant Cyp2e1 protein for bacteriological expression system and the polyclonal antibodies against Cyp2e1. The vector for bacterial expression of the protein was prepared using the following standard methods: computation analysis of corresponding gene primary structure, developing of the primers and PCR conditions, purification of plasmid DNA, restriction, ligation, etc. Purified recombinant Cyp2e1 was also obtained by such standard methods as transformation of bacterial cells, selection of expression and affine purification conditions. For polyclonal antibodies we used immunization of laboratory animals, check of antibody titers, purification of polyclonal antibodies from the sera, and determination of their specificity. The attempts to get full size soluble recombinant Cyp2e1 were unsuccessful due to presence of prolongated hydrophobic region in N-terminal part of the molecule. For this reason the construct pET23-Cyp2e1dBam has been obtained, encoding for 211–493 sequence of the protein. E. coli DE3 cells were transformed with this construct, recombinant protein was purified on Ni-NTA agarose according to manufacturer's protocol (Qiagen). The purity of the preparation was checked with electrophoresis under denaturating conditions (Laemmli). The protein obtained was used for rabbit immunization. Immunization was performed according to the following scheme: 10 subcutaneous injections along spinal column each contained 30 µg of antigen in 100 µl PBS with 50 % Freind's adjuvant, second and third immunizations were done in 9 and 5 weeks, respectively, under the same conditions using incomplete adjuvant. The immune response was observed at 10^{-5} sera dilution. The antibodies from the sera were purified by 2-fold precipitation with ammonium sulfate at 50 % saturation. Further purification was provided in 3 stages: on DEAE-cellulose, protein G sepharose and on affine column with conjugated total E. coli proteins. The affinity of antibodies directed against Cyp2e1 was checked by ELISA, and specificity by Western-blot analysis. In the course of further investigations we are planning to express active recombinant protein Cyp2e1.

Adaptor protein intersectin 1: searching for new protein partners

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Adaptor proteins contain a variety of protein-binding modules that link their partners together and facilitate the creation of large signaling complexes. Linking specific proteins together, cellular signals which elicit an appropriate response from the cell to the environment can be propagated. Thus, adaptor proteins are positioned to regulate cell signaling in a spatial and temporal way. Our investigation was focused on the Intersectin 1 (ITSN1) gene, which is localized on chromosome 21 and encodes a multidomain evolutionally conserved adaptor protein involved in clathrin-mediated endocytosis, cell signaling and cytoskeleton organization. ITSN1 was initially isolated by co-fractionation with dynamin and displayed a particular localization in areas adjacent to the synaptic active zone. A number of synaptic and other proteins were described as intersectin interacting partners. Besides dynamin, ITSN1 was shown to interact with synaptojanin, SNAP-25, Eps15, epsin, SCAMP1, mSos1, Cdc42, CdGAP, N-WASP and other proteins. In our study we identified four new intersectin binding partners – E3 ubiquitin ligases c-Cbl and Cbl-b, Cbl-interacting protein Ruk/CIN85 and microtubule-associated protein 6 (Mtap6). Furthermore, using ITSN1 SH3 domains in pull-down experiments interacting regions were determined. Interaction of ITSN1 with Cbl family ubiquitin ligases and Ruk/CIN85, associated with Cbl, illustrate complicated links between receptor signaling and endocytosis. On the other hand, binding to Mtap6 may involve ITSN1 in regulation of tubulin cytoskeleton that could be important for endocytic vesicle transport. The study was supported by INTAS (project no.05-1000004-7762).

Interferon α and its targets in regenerating rat liver

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IFN-α is a cytokine, known for its antiviral, antiproliferative and immunomodulating effects. However, very little is known about the physiological effects of IFN-α in the absence of viral infection. The mechanisms of IFN-α action in different situations are still obscure. The aim of the study was to investigate the expression of IFN-α, some of its targets (PKR and RNAse L) and IFN-receptors during liver transition from quiescence to proliferation in the absence of viral infection. The rats after partial hepatectomy (PHE) and laporotomy (LAP) were used as corresponding models of G0→S transition and acute phase response. We have used the following methods: PHE and LAP; isolation of the intact liver and liver after 1, 3, 6, and 12 hours after both surgeries; isolation of hepatocytes (Hep) and Kupffer cells (KC) from intact and operated rats; RT-PCR of investigated genes in total liver samples and in isolated Hep and KC; cloning of PCR product of IFN-α into plasmid PUC19 to serve as external control for RT-PCR experiment; the content of IFN in liver samples was evaluated by antiviral test; PKR antigen was analyzed in immunohistochemical and Western-blot experiments. In the samples from intact liver the expression of all genes manifested itself at RNA level. The isolated Hep and KC differ in the amount of specific RNAs. Kupffer cells were responsible for expression of IFN-α but not hepatocytes. The level of IFN-α receptor-, PKR- and RNAse L-specific mRNA was 2-, 3- and 5-fold higher in KC than in Hep, respectively. It corroborates the fact that KC are producers and receivers of cytokines. The PKR protein is expressed in Hep of intact liver and liver during the first 12 hour after PHE and LAP. It appears transiently in KCs in 3 hours after PHE. Immunohistochemical experiments showed the migration of PKR antigen from the nucleus in intact liver to the cytoplasm in the regenerating organ and its reversal to the nucleus at 12 hours post-PHE. After PHE the content of IFN-α protein and mRNA increases in 1 hour after operation with further decrease after 6-12 hours. After LAP IFN-α-specific RNA was detected only using a highly sensitive method of RT-PCR, its content was 2-fold lower than in intact liver, but not detected in the antiviral test. IFN-α specific mRNA was shown to be produced by KC and not by hepatocytes. We assume that IFN-α, cytokine, which belongs to innate immune system, can play its distinct role in the initiation of liver regeneration. It was shown also (Markiewski, 2004) that components of innate immune system, the complement proteins – C3a and C5a – take part in liver regeneration. We consider activation of KC with cell-specific production of IFN-α to be essential for G0 → G1 transition of hepatocytes.

Refolding of ScFv-CBD fusion protein from *Escherichia coli* inclusion bodies

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Overproduction of recombinant proteins in Escherichia coli often leads to their aggregation and formation of insoluble particles in cytoplasm – inclusion bodies. The general strategy for the recovery of functional protein from inclusion bodies involves their solubilization under strong denaturing conditions with subsequent refolding in vitro. However, refolding of recombinant proteins is an empirical process that requires the development of optimized protocol for each individual protein. In case of proteins containing several domains and/or disulfide bonds the task gets complicated due to the requirement for recovery of functional activity for each moiety and/or correct pairing of SH-residues. The aim of this work was to optimize the refolding conditions for insoluble fusion protein ScFv-CBD produced in E. coli. General feature of this fusion protein is presence of three functional moieties, namely, two variable domains from heavy and light chains of antibody molecule (ScFv), that contains internal disulfide bonds, and cellulose-binding domain (CBD). The following methods have been used in this work: isolation and purification of inclusion bodies (centrifugation and immobilized Ni-chelating affinity chromatography), immunoaffinity chromatography, protein SDS-polyacrylamide gel electrophoresis, ELISA, and size-exclusion chromatography. For refolding of ScFv-CBD fusion the earlier developed scheme based on step-wise dilution has been applied. The influence of initial protein concentration on aggregate formation during refolding and final yield of functional ScFv-CBD has been accurately investigated. Strong dependence of molar ratio of glutathione oxidized/glutathione reduced form in refolding buffer for recovery of antigen-binding activity for ScFv-moiety has also been shown. The optimal refolding conditions providing 95 % yield of soluble ScFv-CBD from E. coli inclusion bodies have been determined. The functional activity reached for ScFv- and CBD-moieties was 70 % and 95 %, respectively.

Novel 6,6'-acylamido-disubstituted symmetrical trimethine cyanine dyes for detection of nucleic acids

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Designing of dyes which interact with DNA via groove-binding is actual for the procedures requiring selective detection of double stranded regions of DNA in such diagnostic techniques as quantitating DNA amplification products and detecting DNA molecules in drug preparations. Earlier we have shown that trimethine cyanines are efficient fluorescent probes for DNA detection. Later it was proposed that since the presence of 6,6'-substituents in molecule of trimethine cyanine crescents the shape of molecule it could impart to the dye the preference for groove-binding and thus specificity to dsDNA. Typically symmetric trimethine cyanines with unsubstituted polymethine chain respond slightly to the presence of nucleic acids and thus are inefficient as fluorescent probes for DNA detection. Previously we have shown that insertion of substituent into the 6,6'- position of dye heterocycle allowed us to design the DNA-sensitive dyes on the basis of symmetric dyes, which demonstrated high (up to two hundred times) emission enhancement in DNA presence and extreme DNA selectivity. As a continuation of these studies a series of 6-acylamido-substituted 2-methylbenzothiazoles was synthesized. Starting from them according to standard orthoether procedure a series of 6,6'-acylamido-disubstituted symmetrical trimethinecyanine dyes were obtained. Spectral-luminescent properties of novel fluorescent dyes were studied in unbound state in buffer and in presence of nucleic acids and proteins.

NMR structural studies of cytokine-like C-terminal module of mammalian tyrosyl-tRNA synthetase

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Tyrosyl-tRNA synthetase (TyrRS) catalyzes the activation of L-tyrosine and its coupling to the cognate $tRNA^{Tyr}$. Mammalian TyrRS is composed of two structural modules, namely, the N-terminal catalytic core and C-terminal domain (C-TyrRS). This C-module is highly homologous (52 % sequence identity) to a tumor-derived cytokine EMAP II (Endothelial Monocyte-Activating Polypeptide II) which could induce cell apoptosis, an acute inflammatory reaction and tumor regression in vivo. Recently C-TyrRS was discovered to be a novel cytokine activating endothelial cells and monocytes. In order to elucidate the structural bases of C-TyrRS functions, it is necessary to perform the investigation of its 3D structure in solution by multidimensional high resolution NMR spectroscopy and computational molecular dynamics. The isolated mammalian C-TyrRS was produced as a recombinant protein in Escherichia coli cells using the pEYCD3 plasmid. The ^{15}N and $^{15}N/^{13}C$ double labeled C-TyrRS were prepared by growing the BL21(DE3) strain in M9 minimal medium supplemented with $^{15}NH_4Cl$ and U-$^{13}C6$ glucose as nitrogen and carbon sources, respectively. NMR experiments were performed at 292 and 297 K on Varian Unity+ 500 and Varian VnmrSystem 700 spectrometers equipped with three channels and $^{1}H/^{13}C/^{15}N$ triple resonance probehead. The sequence-specific ^{1}H, ^{13}C, and ^{15}N chemical shift assignments were done on base 3D HNCA and HNCACB spectra. To obtain better spectral resolution these experiments were collected in respect of recently proposed technique of random sampling in evolution time space. Additionally, 3D HNHA and ^{15}N-edited NOESY with a mixing time of 150 ms spectra are also under analysis in order to extract $^{3}J_{HNHA}$ couplings constants and ^{1}H-^{1}H distance constraints to estimate secondary structure of C-terminal module tyrosyl-tRNA synthetase.

Structural and functional comparison of two isoforms of translation elongation factor 1A (eEF1A)

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Translation elongation factor eEF1A is a multifunctional protein which participates in elongation phase of protein biosynthesis. The expression of its isoforms is tissue specific. eEF1A1 is expressed in all tissues except muscles and neurons where eEF1A2 replaces eEF1A1. Recently it has been shown that the eEF1A2 appearance in non specific tissues is accompanied by induction of carcinogenesis. eEF1A2 and eEF1A1 are 97 % homological proteins, and how eEF1A2 expression causes carcinogenesis remains unknown. We have assumed that even insignificant amino acid substitutions in the eEF1A2 primary sequence can influence the conformation of the protein. To test the prediction, we conducted comparative analysis of molecular dynamics of the isoforms in silico. In addition, we compared the conformations of the eEF1A1 and eEF1A2 molecules using differential scanning calorimetry and CD spectroscopy. The heat effect differs considerably for the two proteins, namely, ΔH_{cal} *=580.0 kJ/mol for eEF1A1,* ΔH_{cal} *= 909.4 kJ/mol for eEF1A2. This is an essential difference for two highly homologous proteins. Also, the different value of temperature transition has been observed, namely,* ΔT *= 10.3 °C for eEF1A1 while* ΔT *= = 7.7 °C for eEF1A2. These data mean that two isoforms have different spatial configuration. These observations allowed us to assess the retention of secondary structures of two proteins by comparing their circular dichroism (CD) spectra. The increase in the CD signal for the eEF1A2 at the left side of the absorption spectrum is consistent with a slight loss in* α*-helicity. Thus, molecular dynamic simulation and physical measurements confirm difference in spatial structure of the isoforms. The difference observed leads to differential way of the interaction of the isoforms with various signalling molecules such as calmodulin.*

Obtaining and characterization of single-chain antibodies against recombinant human interferon β1b

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Due to their high binding specificity monoclonal antibodies are widely used in fundamental and applied biomedical researches. The advanced technique for obtaining specific immunological reagents involves generation of large combinatorial cDNA libraries of variable antibody fragments with further selection of recombinant antibodies with desired affinity and specificity. Selection of target molecules from combinatorial antibody libraries is achieved by phage display method providing incorporation of target proteins into the surface of a filamentous phage upon their expression. Phage display allows affinity selection of antigen-specific phages and isolation of target cDNA. The abovementioned technique allows obtaining highly specific recombinant antibodies to a wide range of antigens, as well as ensures their production in prokaryotic cells. The most popular design of recombinant antibody molecules is single-chain fragments variable (ScFv-antibodies), which are widely used nowadays as highly specific immunological reagents. Up to date obtaining of ScFv-antibodies against target antigens is of great practical interest. The aim of this work was to obtain and characterize a panel of mouse ScFv-antibodies against recombinant human interferon β1b (rhIFN-β1b) from an immune combinatorial cDNA library. The following methods have been used in the work: mice immunization, isolation of mRNA, polymerase chain reaction (PCR) with the stage of reverse transcription (RT-PCR), construction of recombinant DNA, cultivation and transformation of E. coli, isolation and restriction of plasmid DNA, DNA electrophoresis, phage display, biopenning of phages, expression of recombinant proteins, ELISA, immunoblotting, protein SDS-PAGE. A cDNA combinatorial antibody library representing $5 \cdot 10^5$ independent clones has been constructed from mice immunized with rhIFN-β1b. The functional size and the initial diversity of the immune library obtained have been determined. As a result of two subsequent rounds of phage affinity selection against immobilized rhIFN-β1b, significant enrichment of the initial library was achieved. Individual E. coli clones producing highly specific ScFv-antibodies against rhIFN-в1b have been isolated. Expression stability for obtained bacterial producers and the yield of functional anti-rhIFN-β1b ScFv have been estimated. High storage stability of the isolated ScFv-antibodies and perfect specificity of their binding to rhIFN-β1b have been shown.

Synthesis of 4-substituted-1-phenylhexahydrothieno[3,4-B]pyrazin-2 (1H)-ones 6,6-dioxide

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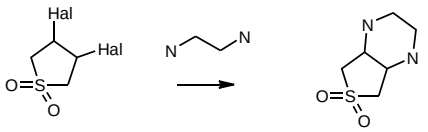
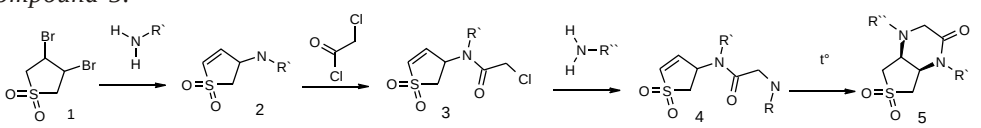
The possibility of obtaining the heterocyclic system hexahydrothieno[3,4-b]pyrazin-2 (1H)-one 6,6-dioxide derivatives was studied with the purpose of searching for new biologically active compounds.Preparation of octahydrothieno[3,4-b]pyrazine 6,6-dioxide by interaction of 3,4-dihalotetrahydrothiophene 1,1-dioxide with excess of ethylenediamine (scheme 1) was already reported.



Scheme 1

Herein we propose a novel and convenient synthetic pathway for preparation of the derivatives of this heterocyclic system (scheme 2), that is impossible to obtain with the methods described before. To obtain the bicyclical system the reaction between compound 3 with primary amines was used. Formation of bicyclical system was found to be staged, and proceeded with intermediate 4 builduping in consequence of primary amine monoalkylation by 3. At higher temperature secondary amino group of intermediate 4 undergoes intramolecular nucleophylic addition to the double bond with formation of bicyclical compound 5.



R` = -C_6H_5, *p*-(CH_3)-C_6H_4, *o*-(CH_3)-C_6H_4, *p*-(H_3CO)-C_6H_4,
R``= -$(CH_2)_{1\text{-}3}CH_3$, -$CH_2(CH)(CH3)_2$, -C_5H_{10}, -C_6H_5, -$CH_2C_6H_5$, *p*-(CH_3)-C_6H_4,
p-(CH_3)-$CH_2C_6H_4$, *p*-(H_3CO)-$CH_2C_6H_4$, *p*-Cl-$CH_2C_6H_4$, -$CH_2C_5H_4N$

Scheme 2

All structures were confirmed by NMR spectroscopy.

Multienzyme conductometric biosensor for direct analysis of sucrose and inhibitory determination of heavy-metal ions

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Development of convenient, precise, selective, fast, and inexpensive methods for determination of different compounds is an issue of the day. In this study, a new conductometric biosensor for sucrose direct determination and inhibitory analyses of heavy-metal ions has been developed and designed. Its sensitive element of two-layer architecture contains three enzymes immobilized onto a conductometric transducer consisting of two pairs of thin-film interdigitated planar electrodes. The biologically active membranes were formed by glutaraldehyde cross-linking enzymes (invertase, mutarotase, and glucose oxidase) with BSA on the surface of one pair of electrodes, while another pair was covered by blank membrane. The characteristics of the biosensor developed were as follows: time of direct measuring sucrose concentration and inhibitory determination of heavy-metal ions in the solution was 1–2 min and 30 min, respectively; linear dynamic range of direct sucrose measurement and inhibitory determination of different heavy-metal ions was 2 μM–5 mM with the detection limit of 200 nM and 0.1 μM–100 μM with the detection limit of 0.1 μM, respectively. Dependence of the sensor response on solution pH and ionic strength was studied. The sequence of metals ions relative to their toxicity toward multienzyme system is $Hg^{2+} > Ag^{2+} > Ni^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Pb^{2+} > Fe^{2+}$*. The developed conductometric biosensor is characterized by high selectivity, operational and storage stability and reproducibility, therefore, it turned out to be suitable for real sample analysis.*

In silico prediction and functional analysis of primary interferon-response genes

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Finding genes which have biologically meaningful ISRE (interferon-stimulated response element) is important for better understanding of the Jak-STAT activated cellular IFN response. We used transcription factor binding site (TFBS) search with gene orthology filtering to find putative ISREs in the promoters of protein-coding genes of Rattus norvegicus, and used Gene Ontology (GO) analysis to check the validity of ISRE search results in terms of biological meaning. A total of 23286 promoters of rat genes were analyzed. To filter biologically insignificant results, we looked for ISRE occurrence in the promoters of orthologues rat and mouse genes, with no more than 25 bp distance between them relative to the TSS (transcription start site) of each gene. GO enrichment testing was carried out using GO Tree Machine (GOTM). ISRE search with 80 % ISRE matrix similarity cut-off produced 5214 sites in 4571 promoters. Of these, 850 ISREs in 768 promoters passed orthology-based selection. Distribution of ISREs along the promoter in 768-gene set reveals 3 regions of ISRE localization: 0 to –250, –250 to –550, and above –550 relative to TSS. It is not yet known whether ISRE localization has any functional applications.GO analysis of 768 gene set versus all rat protein-coding genes produced 48 enriched GO categories ($p < 0.01$), with 13 categories related to known IFN effects. GO analysis of 768 gene set versus 4571 gene set produced 28 categories, with 9 related to known IFN effects. «Cell differentiation» and «development» in biological process, as well as «binding» in molecular function had the lowest p-values in both GO analyses. We identified 768 rat genes which contain ISRE in their promoters, and are the potential targets of transcriptional regulation by type I interferons. Functional analysis of these genes, conducted using Gene Ontology, showed the relative enrichment of some of the GO categories related to already known IFN effects. Additional orthology-based processing of the TFBS search results was shown to produce genes in GO categories, which remain the most significantly enriched when comparing to all-genome protein-coding genes. This indicates that orthology-based selection helps to obtain more biologically significant search results. Genes identified in this research as containing ISRE in promoters will be used to construct the IFN-α-induced gene regulatory network model.

Novel fluorescent dyes for selective fibrillar amyloidogenic proteins detection

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The deposition of insoluble protein aggregates known as amyloid fibrils in different tissues and organs is associated with a number of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. For today there are only several dyes that are used extensively for fluorescent detection of amyloid inclusions, therefore, designing of new dyes which could selectively interact with aggregated into fibrils proteins, is of great importance for basic research. The ability of series of mono- and trimethinecyanines to recognize fibrillar formations of β-lactoglobulin (BLG) and α-synuclein (ASN) as model proteins was studied using method of fluorescent spectroscopy with the aim of searching for novel amyloid-specific fluorescent probes. Firstly it was shown that cyanine dyes could selectively interact with fibrillar proteins giving strong fluorescence responses. The correlation between chemical nature of the dye molecules and ability of dye to bind aggregated proteins was studied. It was shown that incorporation of amino group into the dye molecule could enhance its affinity to amyloid fibrils. Benzothiazole trimethine cyanine with amino substitutes in heterocycle (T-49) demonstrated significant emission increasing upon interaction with fibrillar BLG. Monomethinecyanines with amino- (T-414) or diethylamino- (T-284) substituents showed selective fluorescent response in aggregated α-synuclein presence. Meso-ethyl-substituted trimethinecyanine SH-516 demonstrated fluorescence intensity increase in both aggregated BLG and ASN presence, which even exceeded the corresponding value for commercially available dye Thioflavin T. Such binding preference could be explained by the presence of the styryl fragment in position 5 of the dye molecule heterocycle. Performed calculations of molecular dimensions of studied cyanine dyes allowed us to suppose, that dyes bind with their long axes parallel to the fibril axis via insertion into the neat rows (so called «channels») running along fibril. These studies present a new class of amyloid-specific fluorescent dyes used to develop diagnostic tests for amyloidogenic diseases detection. For the first time it was revealed that tri- and monomethine cyanines can specifically interact with aggregated BLG or/and ASN. Some of these dyes demonstrated higher sensitivity to fibrillar proteins than classical amyloid stain Thioflavin T and could be proposed for application in selective fluorescent detection of aggregated BLG or/and ASN.

Entropy scoring functions for virtual screening purposes

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Today's theoretical knowledge about molecular structure and properties allows developing quality mathematical models of molecular interactions that can be used for prediction of biological activities in pharmaceutical industry. Therefore, it is not surprising that all drug design projects that were recently reported have incorporated the stage of virtual screening. There are two main paradigms for theoretical prediction of biological activity of chemical compounds, namely, ligand- and receptor-based approaches. Due to progress of molecular and structural biology a lot of molecular mechanisms were resolved during last several years. It was followed by the domination of receptor-based approaches in nowadays drug discovery. The receptor-based approaches apply scoring functions – a special sort of mathematical functions that estimates the free energy of molecular system from its coordinates. One of the greatest challenges of today's scoring functions is an accounting of entropy during free energy evaluation. We have developed and implemented the method that performs fast and accurate evaluation of the entropy component of free energy function of a molecular system. Its incorporation resulted in significant increasing of accuracy of the whole virtual screening stage of virtual screening process. The idea of our method is a calculation of relations between various states of molecular system as relations between corresponding volumes of their configuration spaces. The configuration space volume was calculated from the synthetic energy function of the molecular system, which was decomposed in our method up to the second term of Teilor series. The method was successfully tested on the set of resolved crystal structures of molecular complexes of various kinases with their inhibitors. Our method showed especial improvement of activity prediction of compounds, having only small (one or two chemical groups) differences. This makes it particularly promising for activity prediction in the series of organic combinatorial chemistry compounds.

Association of *PMP22* and *Cx32* genes mutations with hereditary motor sensory neuropathy pathogenesis

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Hereditary motor and sensory neuropathy (HMSN), including Charcot-Marie-Tooth (CMT) disease, is one of the most common hereditary disorders, affecting 1:2500 individuals. CMT resulted in mutations of the molecular loci linked to improper regulation of the myelin sheath, axons or cell-cell interactions in the PNS. We have provided the molecular genetic analysis of duplication/deletion of chromosome 17p11.2 including PMP22 gene encoded Pmp22 protein which plays a crucial role in the development and maintenance of compact myelin in autosome dominant HMSN patients. We identified 17p11.2 duplication/deletion using STR markers from 17p11.2 region (D17S122, D17S921, D17S1358, and D17S2226). Cy5-labeled primers and the automated DNA fluorimeter ALF express II were used for fragment STRs analysis. Duplications were found in 19 CMT type 1A families from Ukraine. We detected the deletion in 2 patients of one family with hereditary neuropathy with liability to pressure palsies (HNPP type). The CMTX1 is X-linked type of HMSN associated with mutations in GJB1 (Cx32) gene coding for the gap junction protein connexin 32, which is expressed in Schwann cells. CMTX1 may arise due to incorrect trafficking of Cx32 protein or reduction in the gap junction mediated communication pathway in PNS. Therefore, the next aim of our investigation was screening of Cx32 gene mutation variants. We have developed and applied denaturing gradient gel electrophoresis (DGGE) analysis of Cx32 exons 1B and 2 for suspected X-linked CMT patients. 4 aberrant DGGE-probes have been found. Using direct sequencing the Arg22Gln mutation has been identified in 2 CMTX1 patients.The methods of 17p11.2 duplication/deletion identification and Cx32 mutation variants analysis have been developed in Ukraine. PMP22 duplication/deletion and Cx32 gene mutations have demonstrated association with CMT1A, HNPP, and CMTX1 clinical phenotypes.

A study of the proteins influences on genetic stability of mammalian cells *in vitro*

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Lectins are common carbohydrate-binding proteins of non-immunoglobulin nature. Biological functions of these proteins are different and it is considered that they are determined mainly by their ability to interact with carbohydrates. Protein-carbohydrate interactions take place in various biological processes like the transmission of biological information. Also, exogenous lectins can change such processes as adhesion, migration, apoptosis, and proliferation through the interaction with glycolysed cell membrane, which contains numerous receptors. Thus, the lectins, as a result of their spreading in nature and ability to influence basic matrix processes, are of great interest for conducting scientific investigations with the purpose of their further usage as possible modulators of spontaneous mutagenic process. The aim of our work was to investigate the influence of lectins, which are different in their origin and carbohydrate specificity, on proliferation and genetic stability of mammalian cell populations in vitro. In our experiments we have used lectins of plant (Sumbucus nigra bark, Lens culinaris seeds) and animal (Persa fluviatilis roe) origin, received from NVK Lectinotest, Lviv, Ukraine. All experiments were performed in vitro using Chinese hamster cells line Blld-ii-FAF28C1237. The hprt model was used for investigation of proteins influence on mutagenic process. The scheme of such experiments was described. All investigating lectins have demonstrated the ability to induce mutations in hprt locus in Chinese hamster cells in vitro. In the investigations of their biological action at wide range of doses (from 0.002 to 2000 µg/ml) direct dependence of mutagenic effect on concentration of foreign protein in cellular system has been manifested. Mutagenic activity of lectins is realized according to the last equation and characterized by gradual increasing of mutation frequency with the growth of protein dose. Beside this the antimutagenic effect for combined action of lectins and alkylating agent MNNG on mutagenesis in Chinese hamster cells was revealed. At different schemes of cell treatment by protein and MNNG experimental frequency of mutations induced by two agents was significantly less than theoretical value calculated at the conditions if their independent additive action is expected. We assume that studying mutagens of different nature should use common targets and mechanisms of affect on the cellular DNA.

Authentication of epigenetic changes in genes which are potentially associated with epithelial tumors

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Tumor formation is characterized by both genetic (point mutations, deletions, amplifications, and gene rearrangements) and epigenetic (methylation) changes of cell genome. Presently, the use of biological microarrays is considered to be the most effective among the approaches for authentication of such changes, which assume possibility of early diagnostics of oncologic diseases. The unique technology of DNA-microarrays, based on the use of NotI-linking clones has been developed in the cooperation with Microbiology and Tumor Biology Center, Karolinska Institute, Sweden. This technology allows simultaneous identification of both genetic and epigenetic changes in tumor cell genome and has a number of technological advantages, comparing to other types of DNA-microarrays. It is commonly known that NotI endonuclease recognition sites are mainly localized in CpG-island and the majority of them are associated with 5'-end of genes. Therefore, NotI-microarrays contain clones, which mainly have the sequences of genes, and can be used not only for detecting tumor DNA deletions and amplifications, but also for detecting loss of heterozygosity (LOH) and changes in methylation of DNA. In microarrays we used 182 clones of human chromosome 3, known to contain aberrations in different types of tumors. Screening of the genomic DNA was performed on the samples of surgically separated tumors of epithelial origin, namely, cervical, ovary, colon, and renal. It was established that the most numerous were hemizygous deletions/methylations, less numerous were the cases of amplifications, and the least – the homozygous deletions/methylations. Genetic and epigenetic changes were discovered in a number of genes, involved in carcinogenesis, as well as in genes which can be potentially involved in it. Some of these genes were common to epithelial tumors under investigation and some were observed only in tumors of specific origin. Results of NotI-microarrays analysis for some genes were verified by methyl-specific PCR and methyl-sensitive PCR methods. Comparison of aberrations in the genomic DNA, obtained from the epithelial tumors using DNA-microarrays, methyl-specific PCR, and methyl-sensitive PCR is the beginning of the investigation aimed at discovering groups of molecular changes, which can be used as perspective markers of tumor development processes, especially in early diagnostics. *The work was partly financed by international INTAS grant (Ref. No. 03-51-4983), complex program Novel Medico-Biological Problems and Human Environment, and by grant of the President of Ukraine for the talented young people.*

Cytogenetic studies on the *Gentiana pneumonanthe L.* tissue culture

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G. pneumonanthe L. is European-Caucasian-Siberian species propagated in Scandinavia, Middle and Atlantic Europe, in the North of Mediterranean area, Balkans. In Ukraine it can be occasionally found almost in each botanical-geographical regions (except for Crimea). Employment of G. pneumonanthe for needs of unofficial medicine results in curtailment of number and destruction of its some individual populations. To preserve its natural resources it seems reasonable to employ biotechnological approaches, in vitro culture in particular. It is recognized, however, that application of tissue culture is restricted by high incidence of cell genome changes, affected by the stress from the maintenance on artificial nutrient media. The objective of the work was to study the cytogenetic changes in obtained by us G. pneumonanthe tissue culture. Shoot radicles of the G. pneumonanthe intact plants (Koryukivs'ke forestry, Chernigiv region) and tissue culture of the root origin, derived from it at the 5–9th passages of maintenance, served as purpose subjects. Cytogenetic changes in callus were suggested through comparison of the chromosome number distribution in cultured cells and those in cells of apical meristem from radicles of shoots by examining smash preparations. Concurrently, types and levels of anaphase aberrations were determined. Cytogenetic analysis of the G. pneumonanthe population revealed that the diploid number in the cells of radicle apical meristem equalled 2n = 26. Tissue culture constituted population with the ploidy varying from hypodiploid to hyperoctaploid sets – range of variability for chromosome number comprised from 18 to 110 chromosomes with average number per metaphase being 41. One third of population (33 %) was presented by diploid cells to form the modal class. The fraction of cells with hypo- and hyperdiploid chromosome sets appeared to be rather significant – 7 % and 10 %, respectively. At the same time, the proportion of polyploid cells was high – 50 %, out of which 22 % could be referred to tetraploids. Evaluation of the levels and types of chromosome aberrations in anaphases revealed comparatively low for cultured cells level of mutations – about 7 % of aberrant anaphases, which are presented by single bridges and fragments. Based on the results obtained one can infer, that the maintenance of G. pneumonanthe tissue culture in vitro may result in cytogenetic changes displayed as chromosome number variability and anaphase aberrations. Examination of callus tissues showed their considerable mixoploidy with predominance of diploid and tetraploid cells within the proliferate pool. Potential of the tissue culture for poly- and aneuploidy was established. Chromosome aberration level in anaphases was relatively low.

Gene chitinase 3-like 2 (*YKL-39*) as a potential marker of human glioblastomas

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The aim of this work is to characterize the expression profile of gene YKL-39 in human brain tumors, specifically glioblastomas as compared to normal brain tissue. Chitinase 3-like 2 is a member of the mammalian protein family related in sequence to bacterial chitinases. This protein family includes proteins secreted by human macrophages that have chitinase activity. YKL-39 is very closely related in size and sequence to another member of this family – chitinase 3-like 1 (HC gp-39), and like the latter, as we showed earlier, is upregulated in glioblastomas. The pattern of HC gp-39 expression observed in associations with various pathologies, including arthritis and cancer, indicates a role in inflammation and connective tissue remodeling, as well as in different kinds of cancer. The role of YKL-39 was not enough investigated neither in tumors, nor in normal tissues. To determine gene expression on RNA level we used Northern blot hybridization and reverse transcription-polymerase chain reaction; total RNA for these methods was isolated from frozen tissues according to Chomczyncki and Sacchi; ^{32}P-labeled probes for Northern blot analysis were produced using YKL-39 cDNA fragment. Obtained total RNA was reverse transcribed, using oligo(dT) primer, and amplificated with special YKL-39 primers. To obtain YKL-39 protein we received PCR-product (encoding YKL-39 protein), using specific primers, and cloned it into plasmid in-frame with His-tag sequence. Purified YKL-39 was obtained using Ni-NTA Agarose.Expression profile of YKL-39 on protein level was investigated by Western blot analysis. Rabbit and mice polyclonal antibodies were received using recombinant protein. Serial analysis of gene expression (SAGE) showed that YKL-39 belongs to the pool of genes which are highly upregulated in glioblastoma. Northern blot analysis and reverse transcription-polymerase chain reaction confirmed SAGE data.YKL-39 was obtained and purified from expressed bacterial cells, transformed by special hybrid plasmid. Specificity of the rabbit and mice antibodies was confirmed by Western blot analysis. In the course of these experiments we discovered specific degradation of recombinant YKL-39 in 3 parts. Nature of this hydrolysis is under investigation.

GEF activity analysis of *bcr* Dbl-homology domain

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bcr is a partner of abl in reciprocal translocation between 9 and 22 chromosomes that leads to Philadelphia (Ph') chromosome formation. Bcr-Abl fusion protein is a molecular marker of chronic myelogenous leukemia (CML). Depending on the breakpoints in bcr gene three different size forms of Bcr-Abl protein exist. In 95 % of patients with CML and 30 % of patients with acute lymphoblastic leukemia (ALL) the bcr gene is truncated within major breakpoint cluster region (M-bcr). In 70 % of patients with ALL the breakpoint in bcr occurs in a region upstream of M-bcr named minor breakpoint cluster region (m-bcr). Our research is focused on DH (Dbl homology) domain of Bcr protein which along with PH (pleckstrin homology) domain is located between M-bcr and m-bcr. It is assumed that DH domain has GEF (guanine nucleotide exchange factor) activity against Rho GTPases. We have analyzed previous experiments on this field and established that no investigation was performed on full-length DH domain together with PH domain. Moreover, GEF activity of Bcr DH domain has not been studied in vivo. The object of performed work is analysis of full-length Bcr DH domain GEF activity towards three Rho GTPases in vitro and in vivo. DH domain (481–707 a. a.) of Bcr and tandem of the two domains DHPH (481–869 a. a.) were cloned into pET32a vector for bacterial expression. Purified His-tagged DH and DHPH proteins were used in GDP exchange assay for three Rho GTPases (RhoA, Rac1, and Cdc42). GEF activity of DH domain was determined by labeling each GTPase with [^{3}H]-GDP. DH (or DHPH) protein was added and incubation was performed in 20 mM tris-HCl pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mg/ml BSA at 30 °C. Aliquots were removed and reaction stopped by adding of ice-cold stop solution. Binding of [^{3}H]-GDP was determined by vacuum filtration over nitrocellulose filters and liquid scintillation counting. In vivo GEF activity was studied by GST-pull down assay. 293T cells were co-transfected with DH (DHPH) and GTPase (Rac1, RhoA or Cdc42) in pRK5-myc vector. Cell lysates were incubated with GST-Pak and Rhotekin GTPase-binding domains in the presence of Mg^{2+}. Active GTP-bound GTPase was detected by Western blot. Our data suggest that DH domain of Bcr does not reveal GEF activity towards Rho GTPases in vitro and in vivo. There are evidences that PH domain can facilitate DH functions in some proteins. To study this potential effect we performed the same experiments for DH together with PH domain. There was no significant effect of PH presence on the guanine nucleotide exchange activity of Bcr DH domain.

Development of the methods for DNA diagnostics of most common hereditary disorders using denaturing gradient gel electrophoresis

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One of the main aspects of gene biotechnology is the development and application of effective methods for DNA diagnostics of hereditary diseases (mutation screening). The routine methods are single strand conformation polymorphism (SSCP), heteroduplex analysis (HET), chemical mismatch cleavage analysis (CMC), and denaturing gradient gel electrophoresis (DGGE). DGGE is one of the most powerful methods which allows detecting mutations, including single nucleotide polymorphisms (SNPs), with 100 % accuracy. The DGGE method is based on the principle that the mobility of double-stranded DNA in polyacrylamide gel with linear gradient of the denaturing agents (urea and formamide or temperature) during electrophoresis is retarded by denaturation. A single basepair change will modify the mobility of the fragment and render it detectable. Introduction of a GC-rich sequence through PCR using GC-clamped primers allows the fragment to melt partially and also alters the melting characteristics of the fragment allowing the detection of the mutations in the melted part of the fragment. It has been proposed that the use of GC-clamps among with heteroduplex formation increases the number of detectable mutation variants to virtually 100 %. The aim of our research is the elaboration of the assays using DGGE for analysis of the mutant variants that cause the development of mostly spread hereditary diseases in Ukraine, resulting in early children's death or heavy disability such as cystic fibrosis (CFTR gene) and phenylketonuria (PAH gene). We have performed the design of specific primers and developed diagnostic assays for exons 5, 7, 12 PAH gene, where the most frequent in the Ukrainian population mutations R158Q, P281L, R252W, R261Q, R408W, Y414C are localized. We have also developed diagnostic assays for exons 4, 20 CFTR gene, where mutations W1282X, 621 and R117H were found. The detected mutant profiles were confirmed using restriction analysis. As a result of the DGGE screening in exon 7 PAH gene, the neutral polymorphism V245V was detected. The identified mutant profiles were confirmed using restriction analysis. The unknown mutant variant in the exon 20 CFTR gene was found. To identify this mutant variant the direct sequencing was used. The P1290P polymorphism was identified. Developed methods can be used for creating testing systems for DNA diagnostics in clinical practice, mass and selective screening in genetic testing programs of mutant variants that cause cystic fibrosis and phenylketonuria in Ukraine.

High levels of HC gp-39 protein associated with human glioblastoma development

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Chitinase 3-like 1 gene encoding human cartilage glycoprotein-39 (HC gp-39, also named YKL-40) was revealed by Serial Analysis of Gene Expression (SAGE) among the most upregulated genes in glioblastoma. The cellular responses to HC-gp39 are similar to those elicited by IGF-I. Both proteins act synergistically with respect to their growth-stimulating activity; both suppress the cytokine-induced secretion of MMPs. Besides, these proteins affect tyrosine kinase signaling pathways that include at least four genes, whose activity is increased more than 5-fold in glioblastoma: CD74 antigen, protein tyrosine phosphatase, receptor-type, Z polypeptide 1 (PTPRZ1), EGFR, and chitinase 3-like 1 (cartilage glycoprotein-39). It is not known which signaling pathways are affected in this system, but IGF-1 is a strong activator of PI3K and could exert its protective effects in a similar way to HC-gp39. The results of SAGE analysis for HC gp-39 gene was confirmed by Northern blot hybridization. Western blot analysis of tumor extracts revealed a significant elevation of HC gp-39 protein levels in GB tissue as compared to normal brain tissues. Thus, HC gp-39 plays an important role in malignant progression of astrocytoma and can be used as molecular marker in glioma diagnostics.

Studies of tumor associated antigens from thyroid tumor and melanoma

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The aim of the study was to find and investigate the antigens associated with human melanoma and thyroid tumor. Identification and characterization of TAA from human thyroid tumor and melanoma is an important prerequisite for selection of potential markers for diagnostics of the early tumor stages and monitoring of the course of the disease. The choice of the object for the study was determined by the fact that frequency of the thyroid tumor after Chernobyl disaster increased 2-fold among adults and 3-fold among children. SEREX, construction of plasmid vectors, PCR, affinity chromatography, electrophoresis of the proteins in PAAG under denaturating conditions and nucleic acids in agarose gels, immuno-dot-blot analysis, immunoenzyme assay (ELISA). cDNA expressing library was developed on the basis of thyroid gland carcinoma, amounted 310^6 pfu, with only 2 % of non-recombinant phages. It was sufficient for autologous screening which elucidated 15 immunopositive clones. Among them 5 clones corresponded to the fragments of the gene encoding for catenin-β like protein, two clones encoded for α-catenin and zinc-finger protein NZF, and solid tumors antigen, TOB2, goldgin, Nmc protein and fragments of chromosomes 12 and 6 were presented by single clones. Taking into consideration the results of heterologic screening and analysis of literature data we decided to focus our studies on and β-like catenins. For this purpose the C-terminal fragment of α-catenin and full size gene of β-like catenin were cloned into pGEX4T1 vector, expressed in BL21DE3 E. coli cells and purified with affinity chromatography on GST-sepharose. Recombinant proteins were used for wide scale screening by ELISA method. The preliminary data indicate that part of the sera from thyroid tumor and melanoma patients show high titers of antibodies directed against proteins under investigation.

Expression of eukaryotic elongation factor 1 in human brain tumors

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Elongation factor 1 (eEF1), which is responsible for aminoacyl-tRNA transfer to 80S ribosome, comprises G-protein eEF1A and three subunits of nucleotide exchange factor eEF1B – eEF1Bα, eEF1Bβ, and eEF1Bγ. eEF1A exists as two tissue and development – specific isoforms, eEF1A1 and eEF1A2. eEF1A is a multifunctional protein, related to carcinogenesis. eEF1A1 mRNA is overexpressed in malignant tissues of pancreas, colon, breast, lung and oesophageal as compared to normal counterparts. The protein eEF1A2 has carcinogenic nature in ovary carcinomas and is found to be overexpressed in breast cancer. There are fragmentary facts in literature concerning the overexpression of mRNA for some eEF1B subunits in tumors of different localization but comparative systematic analysis of their expression during carcinogenesis was not carried out. Moreover, absolutely no information is available about expression of different eEF1 subunits at the protein level under carcinogenesis. The aim of this study is to investigate the expression of different subunits of eEF1 in human brain tumors. Using Northern blot analysis we examined the expression level of mRNAs coding for eEF1 (eEF1A, eEF1Bα, eEF1Bβ, and eEF1Bγ) in gliomas (a type of primary central nervous system tumor that arises from glial cells). Northern blot analysis has not revealed any differences in the expression of mRNAs for eEF1A, eEF1Bα, eEF1Bγ in gliomas as compared to normal brain tissues. The eEF1Bβ mRNA expression was not observed. The eEF1Bβ mRNA expression was detected in human gliomas only by RT-PCR suggesting unexpectedly small quantity of this subunit of the eEF1 complex in human gliomas. A very low level of eEF1Bβ mRNA in human gliomas is surprising since the cellular amount of all subunits comprising the eEF1B complex in 1:1:1 stoichiometry should be equal. Previous two-hybrid analysis indicated that the canonical guanine-nucleotide exchange factors eEF1Bα and eEF1Bβ either do not bind or bind poorly to eEF1A2 indicating that eEF1A2, which is brain-specific factor, probably does not need regular exchange factors for its functioning. Novel interaction partner, the muscarinic receptor subtype M4, was recently found to stimulate the exchange of GDP for GTP in the eEF1A2 molecule. We believe the absence of the eEF1Bβ mRNA expression to hint on the possibility of the existence of special GDP exchange factors for the eEF1A2 molecule. Using Northern blot analysis we also investigated the expression level of mRNAs coding for eEF1A1 and eEF1A2 in meningiomas (a benign brain tumor that originates in the cells of the outer covering of the brain (meninges). Two-fold increased level of eEF1A1 mRNA expression and two-fold decreased level of eEF1A2 mRNA expression as compared to the normal brain tissues was observed. Direct measurements of the level of proteins eEF1A1, eEF1A2, eEF1Bα, eEF1Bβ, eEF1Bγ in human brain tumors are being conducted.

Characterization of genes with decrease expression level in human glioma

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The aim of this work is to find genes, which significantly decreased their expression, and may be potential suppressor genes of human brain tumors. Identification and characterization of such genes will supply important information for better understanding of the mechanisms of malignant neoplasm initiation and progression. Definition of differentially expressed genes as molecular markers for determination of distinct molecular tumor subtypes is a crucial challenge for cancer biology. Gliomas, the most frequent and aggressive primary brain tumors, develop through the stages of increased malignancy, associated with the accumulation of genetic abnormalities in different chromosomes and changes of gene expression. A more comprehensive approach to find genes involved in the mechanisms of glioma development requires systematic study of gene expression. Characterization of genes that change their expression in tumors will be exploited for molecular classification of glial tumors, diagnosis and anticancer therapy. Serial Analysis of Gene Expression (SAGE) and Digital Gene Expression Displayer (DGED) as well as Northern blot hybridization and reverse transcription-polymerase chain reaction (RT-PCR) were used to find genes with differential expression in glioblastoma as compared to normal adult human brain. Comparing nine glioblastoma SAGE-libraries and five normal brain SAGE-libraries we found 129 genes with 5-fold changes in expression level, 85 of them were downregulated in glial tumors. The majority of these genes were related to the following groups: genes encoding proteins involved in neurogenesis, extracellular matrix, cell cycle regulation, cytoskeleton, synaptic transmission, transporter activity, endocytosis, signal transduction, apoptosis, ion binding and cell adhesion. Northern analysis of random selected genes confirmed the results of SAGE: DNM1, NRGN and MBP genes, which expressed on the relatively high level in normal brain, significantly decreased their expression in glioblastoma. Since genes FAT2, CPNE6 and NPTX1 have less transcriptional level we used RT-PCR to verify SAGE data and obtained similar results. Characterized genes with decreased expression in glioblastomas as well as genes with significantly increased expression will be used for the creation of test-system (cDNA panel) which will be demonstrated in the report.

Search of the thymidine conformers that are consistent with the low temperature matrix isolation infrared spectrum. An analysis based on DFT and MP2 quantum chemical

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A comprehensive conformational analysis of isolated 2'-β-deoxythymidine (T), canonical DNA nucleoside, has been performed using ab initio calculations at the MP2/6-311++G(d, p)//DFT B3LYP/6-31G(d, p) level of theory. At 298.15 K all the 92 conformers of isolated T are located within a 7.49 kcal/mol Gibbs energy range and in equilibrium distributions syn and S conformers prevail (syn:anti = 61.6 %:38.4 % and S:N = 74.5 %:25.5 %). But at 420 K situation differs essentially (syn:anti = 38.0 %:62.0 % and S:N = 59.5 %:40.5 %). Values of common conformational parameters (P, χ, γ, δ, β, ε, ν_{max}) were established. Energies of the OH...O intramolecular H-bonds as well as their manifestation in ν(OH) stretching vibrations were evaluated. Convolution of calculated IR spectra of all the T conformers appears consistent with its low-temperature matrix spectrum. The maximal discrepancy in frequencies between calculated and experimental spectra is less than 1 %. In general, this result allows reconstructing IR spectra of isolated nucleosides at physiological temperature with rather satisfactory probability. A conclusion was made that dozens of conformers should be considered for reliable reconstruction of the isolated nucleoside IR spectrum.

Investigation on structural-functional role of individual amino acid residues of eukaryotic tyrosyl-tRNA synthetase with site-directed mutagenesis method

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Eukaryotic tyrosyl-tRNA synthetase is composed of two structural modules: NH_2-catalytic module and non-catalytic cytokine-like C-terminal module separated from each other by flexible peptide linker.Unfortunately, full-length eukaryotic synthetase was not crystallized and no information is available about tRNA binding by both full-length and proteolytic modified form of tyrosyl-tRNA synthetase (mini TyrRS). Therefore, the aim of this work is to investigate structural-functional role of some amino acid residues of TyrRS both in tRNA-recognition and in realization of novel non-canonical functions of protein. In 2003 T. Kobayashi et al. obtained and analyzed the structure of Methanococcus jannaschii TyrRS–$tRNA^{Tyr}$–L-tyrosine complex which superimposes well on that of the human mini-TyrRS in its tRNA-free form. It was shown that the first base of anticodon, G34, is flipped out and specifically recognized. The G34 base is sandwiched between Phe261 and His283, and N1 and N2 of G34 are recognized by Asp286 through two hydrogen bonds. His283, Asp286 and Phe261 are well conserved among archaeal and eukaryotic TyrRSs, and correspond to His305, Asp308 and Trp283 of the human TyrRS, respectively. Thus, the specific recognition mechanism of the first letter of the anticodon may be conserved between archaea and eukaryotic. Based on archaea and eukaryotic tRNA homology, and ability of archaea and eukaryotic TyrRSs cross-aminoacylate their tRNAs, we designed a model of complex of B. taurus TyrRS NH_2-module dimer (94 % identity between B. taurus and human mini-TyrRS) with B. taurus $tRNA^{Tyr}$ transcript. During the analysis of the created model the contacts of mammalian mini TyrRS with $tRNA^{Tyr}$ anticodon were identified. The next step was experimental testing of proposed interaction model of TyrRS with $tRNA^{Tyr}$. Therefore, the fragment of cDNA coding NH_2-module of mammalian TyrRS from M1 to L321 was cloned in pBluescript SK(+) vector. Site-directed mutagenesis was performed using QuickChange (Stratagene, USA) method. We replaced His305, Asp308 and Trp283 with smaller amino acid alanine and obtained recombinant clones of mutated proteins in order to study the functional roles of these amino acid residues.