PS4-001

Tissue-specific expression of Arf1 and Sar1 GTP-binding proteins in pea

A. R. Memon1,2, B. Cevher1 and E. Yuca1,2
1Tubitak, Res Inst Genetic Engineering and Biotechnology, 2Biology Department, Yildiz Technical University, Istanbul

Low molecular weight GTP-binding proteins mediate intracellular transport in mammalian and yeast cells. These proteins are about 20–30kDa molecular weight and are important switches that cycle between an active and an inactive state, ensuring vectorial flow of information on the expense of GTP. Arf1 plays an important role in carrying cargo in COPI vesicles in anterograde transport between Golgi cisternae and in retrograde transport from Golgi to ER. Sar1 has a similar molecular weight as ARF1 and is required in anterograde transport from ER to Golgi and is a structural component of COPII vesicles in mammalian and yeast cells. We have identified ARF and Sar in pea plumes. Our data show that Arf1 is mostly concentrated in microsomal and crude Golgi fractions and Sar1 is highly concentrated in crude ER fractions of pea shoots. These proteins were highly expressed in pea shoots compare to roots.

PS4-002

Different role of intracellular loops of glucagon-like peptide-1 receptor in G protein coupling

A. Baveč, M. Hallbrink, U. Langel and M. Zorko
1Institute of Biochemistry, Medical Faculty, University of Ljubljana, Slovenia, 2Department of Neurochemistry and Neurontoxcolgy, Stockholm University, Sweden

The insulinotropic and other effects of glucagon-like peptide-1 (GLP-1) are exerted via the activation of glucagon-like peptide-1 receptor (GLP-1R) that increases cAMP production and also modulates phospholipase C signalling pathway in pancreatic beta-cells and insulinoic cell lines. Previous studies revealed the importance of the third intracellular loop of GLP-1R in coupling to G proteins. In order to study the signalling mechanisms of GLP-1R more closely we synthesized three peptides corresponding to the predicted sequences of the first (IC1), the second (IC2), and the third (IC3) intracellular loop of GLP-1R and we tested their interactions with heterotrimeric G proteins of different types (alpha Gs, Go, Gi1, G11 + beta1 and gamma2 subunits) overexpressed in s9f cells. IC3 powerfully stimulates all types of tested G proteins. Although ineffective when used alone in Rin m5F cells (rat insulinoma cells), IC1 and IC2 show differential effects on G proteins overexpressed in s9f cells and on their activation by IC3. Both IC1 and IC2 activate Gs and also cooperate with IC3 in its stimulation. Go is not affected by IC1 and IC2, Gi1 and G11 are not affected by IC1 but are activated by IC2 which in activation cooperates with IC3. We suggest that GLP-1R is not coupled only to Gs and Gi1, as shown previously, but also to Go and Gi1. IC3 loop is the main switch that mediates signalling via GLP-1R to G proteins, while IC1 and IC2 loops are important in discrimination between different types of G proteins.

Interventions with gene expression

PS4-003

RT-PCR analysis of decorin gene expression in neonatal hypoxic-ischemic rat brain

A. A. Kritis1, E. Spandou1, E. Tsiamitros2 and O. Guiba-Tziampiri1
1Department of Physiology and Pharmacology, Faculty of Medicine, Aristotle University of Thessaloniki, Greece, 2Department of Medical Biochemistry, Faculty of Medicine, University of Thessaly, Larisa, Greece

Introduction: Under hypoxic–ischemic conditions increased deposition of extracellular matrix proteins occurs in various tissues. The aim of this study is to investigate the effect of hypoxia–ischemia on the decorin gene expression in neonatal rat brain.

Materials and methods: Seven-day-old wistar rat pups became ischemic by permanent ligation of the left common carotid. Hypoxia was induced by exposing the pups in an 8% oxygen and 92% nitrogen gas mixture for 60 min at the end of this period and after 2 and 24 h, the animals were decapitated and each cerebral hemisphere was preserved in the RNA-protect solution by Qiagen. Total RNA was extracted from cerebral cortex and hippocampus of both cerebral hemispheres with the RNeasy mini RNA extraction kit from Qiagen. A quantity of 1–2 µg of total RNA were subjected to reverse transcription using 200 units of MMLV-RT (Promega) in 100 µL reaction volume. A volume of 5 UL of the reverse transcription reaction mixture were subjected to competitive PCR amplification under standard conditions including either beta-actin or gapdh as internal controls for gene expression.

Results and conclusions: Here, we report that decorin mRNA is expressed at comparable levels both in the cerebral cortex and hippocampus of the neonatal rat brain. Decorin mRNA levels are of limited abundance compared to those of the beta-actin or gapdh. Although hypoxia–ischemia did not alter markedly the decorin mRNA expression levels induced small changes over the time period investigated. This may be pivotal regarding to the integrity of the extracellular matrix of neonatal rat brain under hypoxia–ischemia.

PS4-004

The EFB and P0 proteins from Aspergillus fumigatus confer resistance to sordarin derivatives in yeast

L. C. Santos, A. Garcia-Marcos and J. P. G. Ballesta
Centro de Biologia Molecular Severo Ochoa, Universidad Autonoma de Madrid, Madrid, Spain

A new family of antifungal compounds derived from sordarin have been described that inhibit protein synthesis. The antibiotics bind to the elongation factor 2 – ribosomal stalk complex and block the translation step. Aspergillus fumigatus, an important human nosocomial pathogen, is resistant to sordarin derivatives. To analyze its resistance mechanism the study was directed to the stalk components (P0, P1, P2) and the elongation factor 2 (EF2B). The stalk components of A. fumigatus ribosomes were biochemically characterized and their cDNAs cloned and expressed in a sordarin sensitive Saccharomyces cerevisiae strain with the P1/P2 endogenous genes deleted and the P0 gene conditionally expressed under the GAL promoter (ScD4567dGP0). P0, P1 and P2 proteins from A. fumigatus are able to form a functional stalk in yeast but the only one conferring sordarin resistance is P0. The A. fumigatus cDNA encoding EFB was cloned in yeast due to the toxicity of this DNA fragment in bacteria. To analyze the effect of the fungal factor, a yeast strain derived from ScD4567dGP0, called E4, was constructed with one of both genes encoding EFB deleted (DefI) and the other regulated by the GAL promoter (GAL:EFT2). The expression of EFB in yeast confers a very high resistance level to sordarin derivatives but the transformed strain grows very slowly. These results indicate that EFB can bind the chimeric ribosomes and form a complex resistant to the inhibitory effect of sordarin. However, the factor is defective regarding its role in translocation.
PS4-005

GM3 synthase mRNA levels in HL-60 cells during monocytic differentiation induced by phorbol esters

E. Sottocornola, B. Berra and I. Colombo
Institute of general physiology and biochemistry, University of Milan, Milan, Italy

During bidirectional differentiation of human myelogenous leukemia cell line HL-60 into monocytic and granulocytic lineages, ganglioside GM3 and neolacto series gangliosides (NeuAc-nLc) are expressed in a differentiation direction-specific manner. That is, GM3 increases markedly during monocytic differentiation of HL-60 cells induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), while NeuAc-nLc increase noticeably in granulocytic differentiation induced by all-trans retinoic acid (RA). These observation suggested that accumulation of particular gangliosides on the cell membrane plays an important role as a trigger in differentiation induction and as determinant of the differentiation direction in some human hematopoietic cells lines. It is known that the critical role played by two key upstream glycosyltransferases, Lc3Cer synthase and GM3 synthase, in regulating the glycosphingolipid biosynthesis in HL-60 cells during bidirectional differentiation, but the mechanisms controlling expression and activity levels of these enzymes have not yet been elucidated. With the present study, according to literature data, we confirm that GM3 synthase is remarkably up regulated during monocytic differentiation, while the GM3 synthase levels did not change in granulocytic differentiation. Furthermore, we demonstrate, by quantitative RT-PCR experiments on total RNA from undifferentiated, RA- or TPA-treated HL-60 cells, the strict correlation between GM3 synthase activity and its mRNA levels. The GM3 synthase transcript is present in equal amount in either undifferentiated and RA-treated cells, but it is drastically increased in TPA-treated cells, suggesting for this enzyme a regulation mechanism at the transcriptional level. Experiments directed to the investigation of Lc3Cer synthase mRNA levels are in progress.

PS4-006

Orphan nuclear hormone receptor Rev-erb alpha regulates the human apolipoprotein CIII promoter

H. Coste and J. C. Rodriguez
Laboratoire Glucosynthyloline, Les Ulis, France

Apolipoprotein (apo) CIII plays an important role in plasma triglyceride and remnant lipoprotein metabolism. Since hypertriglyceridermia is an independent risk factor in coronary artery disease and the presence in plasma of triglyceride-rich remnant lipoproteins is correlated with atherosclerosis, considerable research efforts have been focussed on the identification of factors regulating apo CIII gene expression in order to decrease its production. Here we report on the orphan nuclear hormone receptor Rev-erb alpha regulates the human apo CIII gene promoter. In apo CIII expressing human hepatic HepG2 cells, transfection of Rev-erb alpha specifically repressed apo CIII gene promoter activity. We determined by deletion and site-directed mutagenesis experiments that Rev-erb alpha dependent repression is mainly due to an element present in the proximal promoter of apo CIII gene. In contrast, we found no functional Rev-erb alpha response element in the convergently transcribed human apo AI gene or the common regulatory enhancer. The identified Rev-erb alpha response element coincides with a RORalpha1 element, and in the present study we provide evidence that a functional cross-talk between these orphan receptors modulates apo CIII promoter. In vitro binding analysis showed that monomers of Rev-erb alpha bound this element but not another upstream RORalpha1 response element. In addition, we show that the closely related nuclear orphan receptor RVR also specifically represses the human apo CIII gene. These studies underscore a novel physiological role for members of the Rev-erb family of nuclear receptors in the regulation of genes involved in triglyceride metabolism and in the pathogenesis of atherosclerosis.

PS4-007

Cloning and heterologous expression of archaeal glutamate synthases

H. B. Dinçtürk
Istanbul Technical University, Istanbul, Turkey

Glutamate synthase (GOGAT) is a key enzyme in the early stages of the assimilation of ammonia in a variety of organisms including bacteria, algae and plants, which catalyzes the reductive transamination of the amido nitrogen from glutamine to 2-oxoglutarate to form two molecules of glutamate. Forty-three glutamate synthases differ in molecular weights, subunit compositions and electron donor specificities. The ferredoxin-dependent glutamate synthases found in oxigenic phototrophs are monomeric proteins with Mr of approximately 160–180 kDa. In plants, these enzymes are generally present in green tissues, while the Mr = 220–240 kDa NADH-dependent forms are more abundant in nonphotosynthetic tissues. The bacterial NADPH-dependent glutamate synthases contain two different subunits, with the larger of these subunits showing considerable homology to the monomeric ferredoxin-dependent enzymes. The availability of genome data from several archaea has produced a detailed picture of the evolution of this key enzyme in nitrogen metabolism and the origins of the two subunit/domain structure of the enzyme. We report the cloning, sequencing and the heterologous expression of the putative glutamate synthases from hyperthermophilic archaea: Methanococcus jannaschii and Pyrococcus horikoshii. Two ORFs which show high homology to GOGAT from the other archaea, Pyrococcus horikoshii has also been cloned and sequenced. The 1.5 and 0.8 kb coding regions for M. jannaschii GOGAT kb have been cloned into TA-vectors. The two putative GOGAT reading frames from P. horikoshii are also cloned as above. The bacterial expression studies are carried out in pET vectors.

Nuclear biology

PS4-008

Phosphatidylserine synthesizing enzymes in nuclei from rat liver

A. Dygas, J. Winniewska and J. Baranska
Nencki Institute of Experimental Biology, Poland

PSS1 and PSS2, two base-exchange enzymes synthesize phosphatidylserine (PS) in mammals: PSS1 uses phosphatidylcholine as a lipid substrate and exchanges serine, ethanolamine and choline. PSS2 uses phosphatidylethanolamine and exchanges serine and ethanolamine. PSSs activity was found in endoplasmic reticulum membranes. In cultured cells, both PSSs proteins have been found mostly in mitochondria associated endoplasmic reticulum membranes but in the murine liver subfractions except nuclei. The data suggest that PS should be synthesized in rat liver nuclei rather than by the PSS2 than PSS1 enzyme. To confirm it we use antibodies against 1–17 aminoacids of N-end of PSS1 and 458–474 aminocids of C-end of PSS2. The antibodies recognize appropriate protein bands in nuclei, however, not in other subfractions of the rat liver. In contrast, both antibodies marked very well 42 kDa PSS1 and 52 kDa PSS2 proteins in membranes isolated from various types of cultured cells. These findings suggest, that PSS1 and PSS2 mRNA might be differentially destined to various cellular compartments, or that both proteins might be differentially modified after translation. Thus, aminocid sequences which are recognized by antibodies are (a) both present in PSSs in cultured cells; (b) are absent in rat liver subfractions except nuclei.

PS4-009

Irradiated p53 mutant B-lymphomas reprogram mitotic cell cycle towards endoreduplication coupled to meiosis

J. A. Erenpreisa, M. S. Cragg, A. O. Ivanov, A. Page and T. M. Illidge
1. Latvian University Biomedical Centre, Riga, Latvia, 2. Cancer Sciences Division, Southampton University, UK, 3. Biomedical Imaging Unit, Southampton University, UK

Mitotic failure is known to be a typical response of p53 mutant lymphomas resistant to genotoxic damage. The source of clonogenic survival after such damage is however, less clear. In this study, we have made a number of important observations after a single 10 Gy dose of irradiation: (1) arrest of cells in the spindle checkpoint, which was proportional to radioresistance, with the interruption of anaphase; (2) polar rearrangement of mitotic chromosomes concomitant with the emergence of unipolar spindle; (3) endoreduplication of arrested chromosomes, seen by 3H-T and BrdU inclusion, reaching DNA content up to 64 C; (4) start of endomitotic cycling; (5) formation of the nuclear bouquet structure either from stage (2) or from endophase of stage (4). In cells at stages (4) and (5) mos-protein is abundant, while cyclin B1 is reduced in amount; (6) longitudinal splitting of diploident polyploid bouquets into secondary nuclei concomitantly with segregation of microtubule organizing centres and nucleoli; (7) final segregation of giant cells; down-regulation of mos-protein, up-regulation of cyclin B1; (8) recovery of the mitotically dividing stem-line. We suggest that this switch from mitotic metaphase to endomitosis and meiosis may provide the mechanism to bypass the spindle checkpoint and provide the opportunity for additional DNA damage repair.
Transcriptional machinery

**PS4-010**

**Transcription of SL1, UBF and rRNA genes during lymphocyte mitogenic transformation**

M. Hlavacova and P. Otevrelova

*Institute of hematology U nemocnice 1, Prague 2, Czech Republic*

**Introduction:** Ribosomal genes are transcribed by RNA polymerase I with the assistance of upstream binding factor UBF and promoter selectivity factor SL1, consisting of TATA binding protein TBP and TBP associated factors TAF110, TAF63 and TAF48. Previously, we have demonstrated that mitogenic transformation of quiescent T lymphocytes was accompanied by the promotion of rRNA synthesis and phosphorylation of UBF; both reaching maximal rate after 48 h of pho-tohemagglutinin (PHA) treatment. In contrast, synthesis of UBF mRNA considerably preceded rRNA transcription and increased to the maximal value within 6h. With the intention of elucidating this ‘discrepancy’ we investigated two possible delaying mechanisms: the kinetics of SL1 genes transcription and the kinetics of translation of UBF protein.

**Methods and results:** Human lymphocytes were isolated from the blood by the Ficoll-Paque method. Cells were cultured in RPMI 1640 and stimulated with 10 μg/mL PHA for time intervals up to 72 h. RNA was isolated on InVitrogen’s One-Shot columns and RT-PCR/hybridization was performed using Qiagen one-step RT-PCR kit with specific primers and 32P labeled specific probes. For metabolic labeling, met and cys in media were substituted by 35S labeled analogues. PHA induced transcription of TAF110 mRNA, like TAF63 and TAF48 mRNAs reached its maximal value within 6 h of the start of PHA treatment and correlated with UBF mRNA transcription. UBF protein translation is remarkably delayed relative to its mRNA expression and parallelled the kinetics of DNA replication with maximal rate at 48 h after PHA addition.

**Conclusions:** During mitogenic transformation of quiescent T lymphocytes, UBF and SL1 genes are mobilized and transcribed in the early G1 phase of the first cell division cycle. The early G1 phosphorylation activates UBF pool and rRNA synthesis. UBF synthesis likely occurred at the level of translation starting with S phase and up-regulates S phase ribosomal transcription.

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**PS4-011**

**DSE and SRE binding activity following administration of amphetamine and phencyclidine**

D. Milanovic1, J. Milosevic1, S. Kanazir, L. Rakic2 and S. Rudzic2

*Institute for Biological Research, 1Serbian Academy of Sciences and Art*

Acute administration of d-amphetamine sulphate (AMPH) and (1-[1-phenylcyclo-
heyl]piperidine hidrocloride) (phencyclidine; PCP) produce a characteristic spatio-
temporal distribution of c-Fos protein in the brain. As transcriptional mechanisms underly the induction of c-fos gene expression may be regulated in a stimulus-
specific manner, we have analyzed the binding activities of serum response element (SRE), dyad symmetry element (DSE) and calcium response element (CRE), the major regulatory sites of the c-fos promoter. Electrophoretic mobility shift showed that SRE binding activity was increased for 50–60% in the brain, 2–6h after AMPH, while treatment with PCP resulted in light decrease of SRE binding activity throughout the same time period. Co-administration of AMPH and PCP induced gradual increase of SRE binding activity, reaching maximum (86%) at 6h. Binding of nuclear proteins to DSE sequence was increased 1–2 h after administration of AMPH (72–87%) and remained elevated till the end of the time window observed. PCP and AMPH/PCP caused different temporal profile of DSE binding with peak (40–54%) 4–6h after administration. In contrast, DNA-binding activity of the CRE sequences remained unchanged throughout the time period of 6h under all conditions. Finally, supershift administration. In contrast, DNA-binding activity of the CRE sequences remained unchanged throughout the time period of 6h under all conditions. Finally, supershift analysis clearly demonstrated presence of SRF and c-Fos protein in the transcriptional complexes bound to SRE and DSE sequences irrespective to AMPH, PCP or combined treatment. These finding also showed that the presence of c-Fos protein in the SRE and DSE nucleocomplex support the hypothesis concerning autoregulation of c-fos gene expression during psychostimulant action in vivo.

**PS4-012**

**Nicotine up-regulates expression of p112 mRNA in rat hypothalamus**

A. Yalcin1, O. Konur2, J. K. Kane3 and M. D. Li3

1Department of Biochemistry University of Ege Faculty of Pharmacy Bornova
Izmir, Turkey, Department of Pharmacology University of Tennessee College of
Medicine Memphis, TN, USA

Nicotine is an agonist of nicotinic acetylcholine receptors, which play an important role in neural development, plasticity and excitability. Ubiquitin/proteasome pathway of intracellular protein degradation has been implicated in the involvement of various cellular processes such as cell proliferation and development, cellular stress response, receptor biogenesis, and internalization. Defects in ubiquitin/proteasome pathway were found to be associated with the onset and development of Alzheimer’s and Parkinson’s diseases. p112 is one of the multiple subunits of regulatory particle (PA700) of 26S proteasome. In this present study, we assessed the differences in the expression level of p112 between saline and nicotine treated rats. The brain regions investigated included the brainstem, hippocampus, striatum, amygdala, hypothalamus, cortex, and ventral tegmental area. By using real-time reverse transcription-polymerase chain reaction, we found that mRNA expression of p112 is up-regulated in rat brain in a dose-, time- and region-specific manner in the medial basal hypothalamus. These results suggest that the overexpression of p112 exposure to chronic nicotine promise great potential in enhancing our understanding of the observed association between nicotine usage and neurodegenerative diseases.

**PS4-013**

**Alteration of C/EBP alpha and PCNA association with the rat hepatocyte nuclear matrix throughout development**

S. S. Dinic, S. K. Ivanovic-Matic, M. V. Mihailovic and D. B. Bogojcic

*Institute For Biological Research, Belgrade, Yugoslavia*

CCAAT/Enhancer Binding Protein alpha (C/EBP alpha) transcription factor controls hepatocyte proliferation via the proliferating cell nuclear antigen (PCNA) which expression correlates with cell proliferative activity throughout differentiation. Supporting hypothesis that nuclear matrix (NM) might be involved in control and coordination of nuclear processes during differentiation, we examined localization of C/EBP alpha and PCNA in the NM. In order to assess the degree of these regulatory proteins binding to the NM we compared their relative concentrations in the NM with those in transcriptionally active nuclear extract (NE). Using immuno-western analysis, 30, 35, 38, 42 and 45 kDa C/EBP alpha isoforms were detected in the NMs throughout development. In fetal liver relative concentrations of the isoforms remained unchanged. Birth was followed by increase in the concentration of the 30, 38 and 45 kDa isoforms. In the adult, relative concentrations of all isoforms increased further. Compared to the NM, relative concentrations of C/EBP alpha isoforms in the NEs were significantly lower. In fetal liver only 45 kDa isoform was detected. Until day 7 after birth, C/EBP alpha was represented by 30, 38 and 45 kDa isoforms. By day 14 all five isoforms were detected. Maximal concentrations of all isoforms was observed on the 21st postnatal day when their relative concentrations were higher than in the respective NM pattern and adult NE. Relative concentrations of PCNA in the NMs and NEs were the highest during embryogenesis and decreased until the third postnatal week. PCNA was at the limit of detection in the adult NM and NE. The alternating NM and NE concentrations of C/EBP alpha and PCNA are in agreement with the proposed model that C/EBP alpha stabilizes p21 protein which inhibits PCNA. Established preferential association of C/EBP alpha with the NM could reflect the importance of such interactions for its in vivo functioning. Therefore, partitioning of C/EBP alpha and PCNA in NM and NE could suggest that the regulatory activity of these factors is influenced by their ability to interact with both nuclear compartments.

**PS4-014**

**Two domains of Nrf2 cooperatively bind CREB binding protein and synergistically activate transcription**

K. Itoh1, 2, Y. Kato1, E. Yoshida3, M. Miyagishi, A. Fukumizu and M. Yamamoto

1TARA center, University of Tsukuba, 2Institute of Basic Medical Sciences,
University of Tsukuba, 3Institute of Applied Chemistry, University of Tsukuba,
Tsukuba, Japan

Nrf2 belongs to the Cap-N-Collar (CNC) transcription factor family and is essential for the antioxidant responsive element (ARE)-mediated expression of a group of detoxifying and antioxidant genes. Forced expression of Nrf2 in mammalian cells activates the expression of target genes through the ARE, with Nrf2 showing the highest transcriptional activity among the CNC family of transcription factors. To elucidate the molecular mechanisms generating this potent transactivational activity, we examined the functions of the domains within Nrf2. We found that Nrf2 contains two transcriptional activation domains, Neh4 and Neh5, which act synergistically to attain maximum activation of reporter gene expression. Neh4 and Neh5 both individually and cooperatively bind to CREB (cAMP responsive element binding protein) (CBP). In fact, the specific inhibitor of CBP, adenosine EIA protein, significantly reduced the Nrf2 activity. Importantly, the CBP-binding activity of Nrf2 deletion mutants positively correlated with their transactivational activity. Neh5 contains a motif commonly conserved among the CNC factors, whereas
Neb4 contains the novel CBP-interacting motif recently identified in p53 and E2F. Our results indicate that Nrf2 exploits the cooperative binding of two independent transactivation domains to CBP in the acquisition of a potent transactivation activity.

**PS4-015**

Human choriocarcinoma cell line JEG-3 as a model to study the tissue-specific regulation of cholesterol biosynthesis

K. F. Tacer, S. Kalanj-Bognar, T. Rezen, D. Pompon and D. Rozman

Institute of Biochemistry, Medical Centre for Molecular Biology, Faculty of Medicine, University of Ljubljana, SI-1000 Ljubljana, Slovenia, 2Department of Chemistry and Biochemistry, School of Medicine, University of Zagreb, Salata 3, 10000 Zagreb, Croatia, 3SLPM, Centre de Genetique Moleculaire du CNRS, F91198 Gif-sur-Yvette, France

By investigating the expression of cholesterogenic genes in rat testis we found a discrepancy in expression pattern of these genes. We propose that the lack of a coordinate trancriptional control over cholesterol biosynthetic pathway is the first mechanism leading to meiosis activating sterol from testis (T-MAS) accumulation in testis. In order to study cholesterol biosynthesis in male germ cells, we developed an experimental ex vivo system using cell line JEG-3. We reduced the level of transcription factors sterol regulatory element binding protein (SREBP) that are limiting in male germ cells and activated cAMP-responsive genes of cholesterol biosynthesis through cAMP-dependent pathway. Simulation was not completely successful, since a diminished amount of all analyzed sterol intermediates has been noticed after cAMP-dependent stimulation that is in testis responsible for T-MAS production. The quantity of cholesterol in these conditions decreased, which is in accordance with metabolism of male germ cells that are not efficient in producing cholesterol. Successful sterol repression was observed also on the sterol profile, however, by cAMP-dependent stimuli no change in sterol composition has been observed. Microarray experiments indicate that cell line JEG-3 is not the most appropriate model system for studying cholesterogenic genes due to low expression level of most genes of interest. We propose that low expression of SREBP-responsive genes might be a consequence of high expression level of genes encoding oxysterol producing enzymes. Oxysterols block the production of mature SREBP transcriptional activators which may consequently lead to low-level expression of SREBP-responsive genes.

**Post-transcriptional control mechanisms**

PS4-016

Regulation of papillomavirus gene expression by cis-acting RNA elements

S. Schwartz, B. Collier, D. Oberg, X. Zhao, M. Rush, L. Wållund and A. Grynfeld

Department of Medical Biochemistry and Microbiology, BMC, Uppsala University, Uppsala, Sweden

Human papillomaviruses (HPVs) are small DNA tumor viruses that infect epithelial cells. HPV induces cell proliferation which is required order to replicate the episomal viral DNA. In contrast, terminal cell differentiation is required to express the late genes encoding the two capsid proteins L1 and L2. In vitro this is seen as production of L1 and L2 protein and virus particles only in the upper layers of the epithelium. As a result of the differentiation dependent late gene expression, papillomviruses cannot be efficiently grown in cell culture. The expression of the L1 and L2 genes is blocked in dividing cells. We have identified negative cis-acting RNA elements on the HPV L1 and L2 mRNAs that are active in proliferating cells. Two types of negative regulatory RNA elements have been identified on late HPV mRNAs: elements in the 5' UTR and negative elements in the L1 and L2 coding regions. Results on the regulatory RNA sequences that overlap the L1 and L2 protein coding regions will be presented here. The L1 mRNA contains an inhibitory region within the first 500 nt of the coding region of the mRNA. This sequence acts by dramatically reducing the mRNA levels. Introduction of point mutations that altered the RNA sequence without affecting the protein sequence in this region resulted in efficient production of L1 protein in transient transfections. This region contains multiple inhibitory elements and one of these elements was mapped to a 24 nucleotide sequence in the first 130 nucleotides of L1. Proteins binding specifically to this sequence were identified by UV cross-linking. The negative RNA elements may aid the virus to persist in the infected host. Persistence of high-risk HPV types is a risk factor for development of cervical cancer.

**Post-translational modifications**

PS4-017

Possible role of Streptomyces protein synthesis elongation factor Tu in the cell signalling during differentiation

M. Holub, D. L. Nguyen, O. Benada, O. Kofronova, L. Kalachova, S. Bezouskova and J. Weiser

Institute of Microbiology, CAS, Czech Republic

Protein synthesis elongation factor Tu (EF-Tu) is one of the most abundant proteins found in bacteria. It participates on the correct positioning of incoming aminoacyl-tRNA on the ribosome during translation. There have been proposed several other functions for EF-Tu beside its role in elongation, such as the role in RNA transcription, functioning as a structural protein, the chaperon-like activity or the role in the growth control signalling. These functions seem to be regulated also on post-translation level by the covalent modifications of the protein. To reveal possible role of EF-Tu in signalling cascades involved in the regulation of growth and the differentiation we analyzed content and distribution of the factor in Streptomyces and Mycobacterium cell fractions. We took the advantage of the fact, that although both genera belong to the order Actinomycetales are soil bacteria, only Streptomyces undergo complex process of biochemical and morphological differentiation. EF-Tu sequence data from both genera show that there is a high degree of identity in the large sequence regions. This allowed us to use one of Streptomyces species EF-Tu as a substrate for protein kinase activity analysis in other Actinomycetales species. We compared the heterogeneity of EF-Tu population in Streptomyces grown in liquid and on solid media. Presence of EF-Tu in the membrane fraction of Streptomyces, Mycobacterium and Bacillus was detected by Western blot analysis. In this fraction, we detected protein kinase activity, which phosphorylated EF-Tu in developmentally dependent manner.

PS4-018

Changes in N-glycosylation during yellow lupin seed germination

M. Olczak and W. Wawerek

Institute of Biochemistry and Molecular Biology, Wrocław University, Wrocław, Poland

Acid phosphatase (AP) and diphosphonucleoside phosphatase/phosphodiesterase (PDD 1) were purified from yellow lupin (Lupinus luteus L.) dry seeds, seeds soaked in water for 4 h and seeds after 40 h of germination. Both enzymes are known to differ in type of N-glycosylation pattern typical for vacuolar protein, the second enzyme – typical for extracellular or membrane bound protein. N-glycans were released from the each enzyme preparation, fluorescence labeled, separated and identified in HPLC (Glyco Sep N and Glyco Sep H columns). Changes in a level of the each released and separated glycan occurring during germination (0, 4 and 40 h) were compared. The results show that N-glycan biosynthesis and processing in case of AP and PDD 1 – two proteins residing in the same plant organ (seed) is not
synchronized. The increase of high mannose glycans level (accompanying always a new glycoprotein biosynthesis) is much earlier and higher in case of AP. The increase of high mannose glycans level (accompanying always a new glycoprotein biosynthesis) is much earlier and higher in case of AP.

**PS4-020**

**Yeast Cu-Zn superoxide dismutase as an inhibitor of ribosomal P proteins phosphorylation**

R. Zielinski1, M. Pilecki1, K. Kubinski1, P. Zien1, U. Hellman2 and P. Zielinski1

1Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poznań, Poland, 2Center for Controlled Chemical Delivery, Department of Pharmaceutics, University of Utah, 570 Biomedical Polymers Bldg., 10205, Salt Lake City, Utah 84112, USA, 3Center for Complex Molecular Systems and Biomolecules, Flemingovo nam. 2, 16610 Prague, Czech Republic

The mature cathepsin C molecule is composed of four identical monomers each proteolytically processed into residual propart and two-chain catalytic core. Three nonpaired cysteine residues per monomer were identified. Beside catalytic Cys234 in the active site, free-thiol Cys331 and Cys424 were characterized. Cys424 was classified as inaccessible buried residue. Selective modification of Cys331 resulted in dissociation of native cathepsin C tetramer into dimers. The 3D model of the cathepsin C molecule suggests that Cys331 becomes exposed as the activation peptide is removed during activation of cathepsin Czymogen. Cys331 is a part of the dimer-dimer interaction interface involved in the assembly of two zymogen dimmers. The structural rearrangement of this region triggered by maturation processing of preprocathepsin C and catalytic core is discussed. Supported by the grants GA AV CR A4055006, GACR GP2030/01/D008 and 522000/1553, and project Z4055/905.

**PS4-022**

**Identification of N-glycosylation sites critical for the activity of recombinant human glutamate carboxypeptidase II**

C. Barinka1, P. Sacha1, J. Sklenar2, K. Bezouska2, B. S. Slusher2 and J. Konvalinka1

1Institute of Organic Chemistry and Biochemistry, Flemingovo no. 2, Prague, Czech Republic, 2Department of Biochemistry, Faculty of Natural Science, Charles University, Hlavova 30, Prague, Czech Republic, 3Guilford Pharmaceuticals Inc., 6610 Tributiary Street, Baltimore, MD 21224, USA

Glutamate carboxypeptidase II (GCPII, EC 3.4.17.21) is a membrane peptidase expressed in a number of tissues such as prostate, small intestine, tumor-associated neovascularization, and central nervous system, where it cleaves N-acetyl-L-aspartyl-L-glutamate (NAAG) yielding free glutamate in the synaptic cleft. Animal model experiments show that inhibition of GCPII prevents neuronal cell death during experimental ischemia. GCPII thus represents an important target for the treatment of neuronal damage caused by excess glutamate. GCPII is a glycoprotein, and 10 potential N-glycosylation sites were predicted for human GCPII. A system for recombinant expression of milligram quantities of human GCPII in Drosophila Schneider’s cells was developed and novel assay for GCPII hydrolytic activity, based on fluorometric detection of released alpha-aminoo groups, established. To identify the domain(s) responsible for GCPII proteolytic activity, N- and C-terminally truncated GCPII variants were constructed and their respective NAAG-hydrolyzing activities analyzed. Finally, we report loss of NAAG-hydrolyzing activity after GCPII deglycosylation and in cells treated with tunicamycin, a potent inhibitor of N-glycosylation. To clarify the role of individual N-linked oligosaccharides for GCPII carboxypeptidase activity, series of mutants (Asn to Ala) with single or double mutations in N-glycosylation consensus sequences were generated and catalytic activity of the mutated proteins examined. Two N-glycosylation sites distinct from the putative active site of the enzyme were shown to be critical for NAAG-hydrolyzing activity of GCPII.

**PS4-023**

**Hydrophobic interaction chromatography of human alpha-thrombin**

G. Karlsson

Analytical Chemistry, Plasma R&D, Biovitrum

Human alpha-thrombin is a plasma serine protease that converts fibrinogen to fibrin monomers, activates Factor XIII, initiates platelet secretion and aggregation, is involved in the activation of protein C, is inhibited by antithrombin, and thus plays a crucial role in blood coagulation. The major active form of human thrombin, alpha-thrombin, was analyzed by means of a hydrophobic interaction high performance liquid chromatography (HIC-HPLC) system, including a TSK Phenyl 5PW column (75 mm x 7.5 mm ID). The mobile phases used for elution were, 2 mM sodium chloride, 25 mM Tris–HCl buffer, pH 8.0 (mobile phase A), and 25 mM Tris–HCl buffer, pH 8.0 (mobile phase B). A linear gradient was run (0–5 min 0% B, 5–20 min 0–100% B, 20–30 min 0% B). Flow rate was 1 mL/min. The sample, 0.05 mL thrombin (0.5 mg/mL) was injected, and detection was carried out by measuring the absorbance at 280 nm. By this method, a good resolution between alpha-thrombin and the theoretically modified thrombin forms, beta- and gamma-thrombin, was obtained. In addition, the thrombin preforms, prothrombin, prothrombin 1 and 2, were also resolved from alpha-thrombin in the system. The results from the HIC method were in concordance to those obtained from nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis. By using this high-resolution chromatographic method, a rapid analysis of purified alpha-thrombin is obtained. In addition, the method could be up-scaled in the development of purification procedures for alpha-thrombin.
**PS4-024**

**Influence of shear stress on the expression of tyrosylprotein sulfotransferases in human endothelial cells**

S. Goettsch, W. Goetsch, H. Morawietz and P. Bayer

MPI For Molecular Physiology, Dortmund, Germany

The sulfation of protein tyrosine residues is a widespread post-translational modification, which influences protein–protein interactions, enzymatic activity and protein life-span, affecting processes like inflammation and blood clotting. The O-sulfation of tyrosine residues is catalyzed by tyrosylprotein sulfotransferases (TPST). We investigated the expression of the two human TPSTs in human umbilical vein endothelial cells. These cells are constantly exposed to shear stress and respond to that besides other activities by releasing proteins and by changing gene expression. For the first time coexpression of TPST1 and two within a single cell type could be shown. After application of physiological levels of shear stress the dominant TPST isoform was shifted in a time- and dose-dependent manner as validated by quantifying mRNA and protein expression. Arterial laminar shear stress decreased the expression of TPST1 mRNA and protein to 67 and 66% of control without shear stress after 24 h of fluid flow. In contrast, expression of TPST2 mRNA is stimulated up to 176% after 4 h already. The independent response of the isoforms to shear stress could be further validated. Down-regulation of TPST1 could be prevented by an inhibitor of tyrosine kinase signalling pathway, whereas the increase of TPST2 after application of shear stress is completely blocked by inhibition of the protein kinase C pathway. In conclusion, endothelial cells are able to modulate the expression of tyrosylprotein sulfotransferases via specific phosphorylation events. An alternative TPST isoform might lead to tyrosine sulfation of a different subset of proteins and might, therefore, influence a variety of intra- and extracellular processes.

**PS4-025**

**Advanced glycation end products on elastin: the effects of Fe(II) and Cu(II) ions**

S. Civelek and G. Burcak

Department of Biochemistry, Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey

The process of advanced glycation during aging and at an accelerated rate in diabetes mellitus modifies the extracellular matrix proteins. Increased stiffness and resistance to proteolysis are the important features of this modification. Despite its importance in the cardiovascular system, lungs and spine scant information is available on advanced glycation of elastin. The aim of this study was to develop an *in vitro* model system to study advanced glycation of elastin and to investigate the effects of high concentrations of Fe(II) and of tyrosine kinase signalling pathway, whereas the increase of TPST2 after application of shear stress is completely blocked by inhibition of the protein kinase C pathway. In conclusion, endothelial cells are able to modulate the expression of tyrosylprotein sulfotransferases via specific phosphorylation events. An alternative TPST isoform might lead to tyrosine sulfation of a different subset of proteins and might, therefore, influence a variety of intra- and extracellular processes.

The problem of advanced glycation during aging and at an accelerated rate in diabetes mellitus modifies the extracellular matrix proteins. Increased stiffness and resistance to proteolysis are the important features of this modification. Despite its importance in the cardiovascular system, lungs and spine scant information is available on advanced glycation of elastin. The aim of this study was to develop an *in vitro* model system to study advanced glycation of elastin and to investigate the effects of high concentrations of Fe(II) and Cu(II) ions. Elastin has been isolated from thoracic aorta and elastase from pancreas of young calves. Incubation groups were designed as 'Elastin', 'Elastin + Glucose', 'Elastin + Glucose + Fe(II) or Cu(II)', 'Elastin + Glucose + DETAPAC', 'Elastin + Glucose + DETAPAC + Fe(II) or Cu(II)', 'Elastin + Fe(II) or Cu(II)'. The concentrations of glucose were held at 278 mM, Fe(II) 1.8 mM, Cu(II) 2 mM and as a metal chelator diethylenetriaminepentaacetic acid (DETAPAC) 1 mM. The AGE compound released with elastolytic activity was analyzed with spectrofluorometry. In all incubations, a brown product with fluorescence characteristics of a typical AGE-compound with excitation at 370 nm and emission at 440 nm was observed to accumulate on elastin. The addition at high concentrations of neither Fe(II) ions nor Cu(II) ions caused an increase in advanced glycation of elastin but chelation of the metal ions caused a significant decrease. An *in vitro* model system has been established to study advanced glycation of elastin and it has been shown that advanced glycation accelerated by trace amounts of Fe(II) and Cu(II) ions is not further accelerated by the high concentrations.

**PS4-026**

**Palmitoylation of apoB improves ER-Golgi sorting, lipoprotein particle biogenesis and secretion efficiency**

G. L. Vilas and L. G. Berthiaume

Department of Cell Biology, University of Alberta, Alta., Canada T6G 2S7

Apolipoprotein B (apoB), an essential component of various low density lipoproteins and chylomicrons, is a palmitoylated protein. To investigate the role of palmitoylation in lipoprotein particle assembly, a palmitoylation site was mapped to Cys1085 in a truncated apoB variant (apoB-29) and removed by mutagenesis [1]. The secreted lipoprotein particles formed by the nonpalmitoylated apoB-29 were smaller, denser and contained 80% less cholesterol ester (CE) and triglycerol ester (TG) than wild type controls. In the present work, we show that palmitoylation of apoB: (1) occurs early in its biogenesis (ER compartment); (2) improves ER-Golgi transport rate (wt apoB-29 1/2 = 26 min while Cys1085Ser mutant t1/4 = 46 min); (3) improves secretion efficiency (wt = 53%, mutant = 26%); and (4) appears to act as a quality control step favoring dissociation of wt apoB from ER chaperones calnexin (CNX) and UDP glucosyl glucosyl transferase (UGGT). In addition, we show that nonpalmitoylated apoB-29 colocalized extensively with constitutively secreted protein transferrin while wt apoB-29 showed only partial colocalization and appeared to be enriched in extensions of the ER. Our results suggest that palmitoylation of apoB actively regulates the biogenesis of apoB-containing lipoprotein particles by concentrating apoB in a specialized ER compartment and by favoring dissociation with ER-localized chaperones (CNX and BCA) and reducing interaction with the ER-Golgi transit time and higher secretion efficiency of wt apoB-29. Regulation of apoB degradation by palmitoylation (perhaps via palmitoyl-CoA levels) could thus be regulating the amount of apoB available for secretion of CE and TG in the blood.


**PS4-027**

**Activation of tumor suppressor protein p53 to DNA binding by post-translational modifications**

S. Popisilova1, V. Brazda2, K. Kucharikova2,3, P. Skladal1, E. Palecek2 and B. Vojtesek1

1Masaryk Memorial Cancer Institute, Zluty kopec 7, Brno, Czech Republic,
2Institute of Biophysics, Academy of Sciences of the Czech Republic, Kralovopolska 1/55, Brno, Czech Republic, 3Department of Biochemistry, Faculty of Science, Masaryk University, Kolariska 2, Brno, Czech Republic

The tumor suppressor protein p53 is the potent transcription factor playing a key role in cell cycle regulation triggering growth arrest or apoptosis in response to different cellular stress signals. The p53 protein is structurally flexible and can adopt the latent, non-DNA binding, or the active, DNA binding conformation which is crucial for the transactivation function of p53. The activation of latent p53 to DNA binding can be induced by targeting its C-terminal regulatory domain. The post-translational modification of specific sites within this region, mainly phosphorylations, are supposed to play an important role in this process. We studied the mechanisms of latent p53 protein activation to sequence-specific DNA binding by phosphorylation with cdk2/cyclinA at serine 315, with protein kinase C at serines 371/373 and with casein kinase II at serine 392. Using nonradioactive EMSA we showed, that both cdk2/cyclinA and PKC phosphorylations were capable of efficient activation of the latent p53 protein binding to the DNA fragment carrying the p53 binding element. Similar effect provides the p53 interaction with monoclonal antibody Bp53–10 recognizing the C-terminal epitope of the protein. Surprisingly, the CKII phosphorylation induced the DNA binding activity only in low extent and, in addition, the presence of CKII phosphorylation inhibited the PKC-induced activation of p53–DNA binding function. The results indicate the crucial role of C-terminal post-translational modifications of p53 in the activation of protein–DNA binding function but also suggest the antagonistic relations between different stress signalling pathways. This work was supported with grants no. 301/00/P094 and 301/02/0831 from GACR.

**PS4-028**

**Molecular mechanism of tyrosine phosphorylation of autokinase WZC in Escherichia coli**

C. Grangeasse, P. Doublet, B. Ohabia and A. J. Cozzone

Institute of Biology and Chemistry of Proteins, Lyon, France

In bacteria, several proteins have been shown to autophosphorylate on tyrosine residues but little is known on the molecular mechanism of this modification. To get more information on this matter, we have analyzed in detail the phosphorylation of a particular autokinase, protein Wzc, from *Escherichia coli* K12. The analysis of the hydrophatic profile of this protein indicates that it is composed of two main domains: an N-terminal domain including two N-terminal trans-membrane helices and a C-terminal cytoplasmic domain. The C-terminal domain alone can undergo autophosphorylation and thus appears to harbor the protein-tyrosine kinase activity. By contrast, the N-terminal domain is not phosphorylated when incubated either alone or in the presence of the C-domain, and does not influence the extent of phosphorylation of the C-domain. The C-domain contains six different sites of phosphorylation. Among these, five are located at the C-end of the molecule in the form of a tyrosine cluster (Y708, Y710, Y711, Y713, and Y715), and one site is located upstream, at Y569. The Y569 residue can autophosphorylate through an intramolecular process, whereas the tyrosine cluster cannot. The phosphorylation of Y569 results in an increased protein-kinase activity of Wzc which can, in turn, phosphorylate the five terminal tyrosines through an intermolecular process. It is concluded that protein Wzc autophosphorylates by using a cooperative two-step mechanism which involves both intraphosphorylation and interphosphorylation. This mechanism may be of biological significance in the signal transduction mediated by Wzc.

**PS4-029**

**Design, construction and thermodynamic characterization of highly stable human acidic fibroblast growth factor**

M. Zakrzewska, D. Kowarszch and J. Otrowski  
*Institute of Biochemistry and Molecular Biology, University of Wroclaw, Poland*

Human acidic fibroblast growth factor (FGF-1) is a powerful mitogen, inducing the proliferation of a wide variety of cell types, and playing an important role in morphogenesis, angiogenesis and wound healing. FGF-1 is inherently poorly stable protein and the low stability of the wild type of FGF-1 may be related to regulatory mechanisms, especially cell membrane translocation. FGF-1 has a b-trefoil structure with a centrally located hydrophobic internal cavity. Using molecular modeling and homologue sequence and structure comparisons of 48 selected members of the FGF family, we designed and constructed five point mutants of FGF-1 and defined their thermodynamic stability. The mutations (H121Y, L44F, H102Y, F108Y, V109I) were introduced by site-directed mutagenesis, expressed using the pET-3c plasmid/Bi21(DE3)pLysS host expression system and purified by affinity chromatography. The aim of the residue replacements in the FGF-1 molecule was to optimize the packing of the protein (by central cavity rearrangement) or to create new hydrogen bonds and salt bridges to stabilize the protein structure. The effects of the mutations were determined by differential scanning calorimetry, circular dichroism and fluorescence spectroscopy. A proper fold of the constructed mutants was examined before thermodynamic stability studies. We determined (by chemical and temperature denaturation) that all the induced mutations increased the stability of FGF-1. The most stable variant of FGF-1 has a free enthalpy of denaturation 1 kcal/mol higher than the wild type of the protein.

**PS4-030**

**Influence of spermidine on callus formation and somatic embryo production from Helianthus annuus L. hypocotyl protoplasts**

A. Auron1,2, S. Ghenescu3,4 and L. R. Tican1,2  
1Plant Genetic Manipulation Group, Faculty of Biology, Babes-Bolyai University, 2Department of Ecology-Genetics, Plant Genetic Manipulation Group, Faculty of Biology–Geology, 5–7 Clinicior Street, 3400 Cluj-Napoca, Romania

Genetic improvement of cultivated sunflower (*Helianthus annuus* L.) is a necessity due to its importance as oil crop in the world. Regeneration of fertile plants from sunflower protoplasts is very difficult in spite of numerous attempts made to stimulate protoplast regeneration through biotechnological techniques. We describe here the stimulation of protoplast regeneration to callus and somatic embryos by using the polyamine spermidine in culture medium. Hypocotyls of *Helianthus annuus*, Romanian cultivars Turbo and Select, grown in vitro for 7 days, in dark, on MS/1/2 medium, were used as source of protoplasts. The enzyme mixture was according to Krasnyanski and Menczel (1993), protoplasts were plated at 10 × 10 000 cells/mL, immobilized in alginate disks and cultivated in L4 liquid medium supplemented for half of probes with 1 μM spermidine. Plating efficiency (number of cells dividing of total number of cells) was calculated at 10 and 20 days of culture on medium with and without spermidine. After 4–5 weeks small calli were observed at both Turbo and Select, and somatic embryos in all developmental stages at Turbo, but they failed to develop into plantlets. The present data suggest that spermidine has a positive role in somatic embryogenesis in early stages of development.

**PS4-031**

**Differential effects of P2Y1 and P2Y12 receptors activation on signaling pathways in glioma C6 cells**

R. Czajkowski1, W. Banachewicz1, O. Inytska2, L. Drobot2 and J. Baranska1  
1Department of Molecular and Cellular Neurobiology, Laboratory of Signals Transduction, Nencki Institute of Experimental Biology, Warsaw, Poland, 2Department of Cell Signalling, Institute of Cell Biology, Lviv, Ukraine

We have shown that glioma C6 cells express two types of nucleotide ADP receptors: P2Y1 and P2Y12. P2Y1 is coupled to phospholipase C (PLC) activation and mobilization of intracellular calcium as well as protein kinase C (PKC) activation. P2Y12 receptor is coupled to Gi protein and inhibition of adenyl cyclase. ADP, 2-MeS ADP and 2-Cl ATP act as agonists of both receptors. MRS 2179 acts as specific antagonist of P2Y1. This allows to distinguish between biological effects evoked by both receptors. We have demonstrated that ADP receptors agonists stimulate ERK1/ERK2 kinases activity in glioma C6 cells. The stimulation is transient, reaching the peak at 5 min after agonist addition, and declining to the basal level within 1 h. MRS 2179 only partially affects this activation. Inhibition of calcium signal evoked by P2Y1 also has only a partial effect on this process. This suggests the involvement of both, P2Y1 and P2Y12 receptors in ERK activation. We also investigated the effect of ADP on PI 3-kinase activity. We found that ADP can evoke differential effects on PI 3-K, depending on culturing conditions. In the cells cultured in the presence of serum, where the basal level of the enzyme activity is high, ADP inhibits PI 3-K. In the starved cells, basal activity of PI 3-K is low, and ADP stimulates the enzyme. This increase in enzyme activity occurs within 15 min after stimulation, when calcium signal is terminated. Treatment with MRS 2179 enhances the stimulatory effect of ADP. GF 109203X, an inhibitor of PKC, that increases calcium response after P2Y1 stimulation, diminishes this effect. These data provide an indirect evidence that P2Y1 is responsible for inhibition, and P2Y12 for activation of PI 3-kinase after ADP treatment.

**PS4-032**

**Downstream AKTion of PI three kinase**

A. K. Froese1, H. Holmsen and N. Aarsaether  
Department of Molecular Biology and Biochemistry, University of Bergen, Norway

The phosphatidyl inositol three Kinase (PI three kinase) pathway plays an important role in regulating cellular proliferation and mutations in components of this pathway may have implication in carcinogenesis. AKT, also referred to as PKB, is a downstream target of PI three Kinase and plays a critical role in controlling the balance between survival and apoptosis. This protein kinase is activated by insulin and various growth factors and functions in a wortmannin sensitive pathway involving PI three kinase. In order to investigate the molecular implications of PI three kinase and AKT pathways on the mRNA expression relevant to insulin signalling, we have preformed expression profiling using cDNA macroarray on human neuroblastoma cell line. One of the mRNAs that was strongly up regulated in this study was insulin growth factor binding protein-two (IGFBP2). IGFBP2 belongs to a family of structurally related binding proteins. Six human insulin growth factor binding proteins are known (IGFBP one to six), which bind IGF I or IGF II with different affinity. IGFBPs modulate IGF action at cellular level through proteolysis and are important in the control of neuroblastoma cell proliferation. IGFBP2 is known to be a highly sensitive marker for malignant progression in different tumors, but the mechanism by which IGFBP2 might affect tumorigenesis is largely unclear. By using a specific MEK inhibitor and wortmannin, a specific inhibitor for PI three kinase, and applying real-time PCR we are now currently investigating the role of these pathways in insulin signalling relevant to IGFBP2 mRNA expression.

**PS4-033**

**Characterization of DGK alpha as a novel PI3K effector**

A. Cipres-Blanco, M. A. Sanjuan and I. Merida  
Department of Immunology and Oncology, National Center of Biotecnology, Madrid, Spain

Diacylglycerol kinase (DGK) alpha converts diacylglycerol (DAG) to phosphatidic acid (PA). DGK contains two EF-hands suggesting a putative regulation by calcium and calcium regulation has been demonstrated in response to T-cell receptor. The enzyme also contains two atypical cistein-rich motif of unknown function. Nevertheless, DGK can also be activated by receptors that do not induce short-term increases in DAG or calcium levels, i.e. IL-2 or insulin receptor. In this study, we have used a stable clone of Ba/F3 cell line that express the heterotrimeric IL-2R to look for novel mechanism of activation. We show that DGK is activated by the LCK tyrosine kinase by a mechanism dependent on PI3K activity. Moreover, the expression of a constitutive active form of PI3K also increases an increase in DGK activity. When this form of PI3K, as well as the active construct of LCK, are expressed, DGK translocates from cytosol to plasma membrane. Using in vitro assays we demonstrate that the products of PI3K bind and activate DGK. By generating different truncations and mutations of DGK we show that the cysteine-rich domains are the motifs responsible for PI3K dependent regulation of DGK. Finally, we show that the inhibition of PI3K, as well as LCK, abolishes the IL-2-induced DGK activation. Altogether, these studies demonstrate for
the first time the existence of a novel pathway where, in the absence of receptor-regulated PI turnover, DGK regulation is a direct consequence of PI3K activation.

**PS4-034**

Erythropoietin receptor in fetal and neonatal pigs

R. B. David, A. K. Blom, I. Harbitz and O. V. Sjaastad
Norwegian School of Veterinary Science, Norway

The erythropoietin receptor (EpoR) is a single transmembrane protein, two of which must be bound to erythropoietin (Epo) to achieve transduction. The kidney is the major site of Epo production in the adult mammal, whilst the bone marrow is the major site of erythropoiesis. In the fetus, the liver is the major site of both Epo production and erythropoiesis. In order to determine when erythropoiesis in the pig migrates from the liver to bone marrow, real-time RT-PCR was used to quantitate EpoR mRNA levels in these organs throughout development (term = 115 days). Samples from the spleen and were also tested because it possibly has an erythropoietic function. Compared with the expression levels in the liver at birth, the mean levels of EpoR gene expression in the liver at 30, 50, 70, 100 and 110 days gestation were: 8.6, 8.5, 6.5, 1.9 and 2.5, respectively. At 14 and 35 days after birth, the levels were 0.4 and 0.6, respectively. Compared with the expression in the bone marrow at 70 days of gestation, the mean levels at 14 and 35 days of age were 4.3 and 4.2, respectively. The spleen had a high expression of EpoR just before birth. We conclude that the transition takes place near the time of birth, and the spleen helps to ease this transition by taking up the slack.

**PS4-035**

Possible reciprocal action of IGF-1 and thyroid hormones

A. Karus and V. Karus
Estonian Agricultural University

The aim of the present study was to investigate interactions between insulin-like growth factor-1 (IGF-1), triiodothyronine (T3) and thyroxine (T4) in different stages of cattle development. Growth hormone and IGF-1 are general growth controlling factors, but they play also an important role in the animal development. In our study thyroid hormones showed higher values on the second postnatal month. However, their level remains relatively stable during first year. At the end of second month the T3 level was 152 ± 8 ng/dL and T4 level 5.24 ± 0.18 µg/dL. We observe a weak tendency to decrease of T3 level in the second half of the first year. The T3 value in serum achieves at the end of first year an average 22% under the maximum value for this individual animal. T4 loses only 3% of its maximum value. In the same period the content of IGF-1 in serum shows the tendency to increase, but the maximum value achieved in 13–14 month age. The content of IGF-1 in this period was 180 ± 7 µg/L. That can be connected with the cell differentiation in this period. Moreover, the IGF-1 level in bovine serum can be predicted from a linear equation of the animal age (in days) and T4. CIGF-1 apoptosis irrespective of cell type, in an SRF-dependent manner. We further present evidence that Elk-1 has a role in neuronal differentiation in PC12 model system. We propose that TCFs act as key factors in regulating cell survival and/or protecting from apoptosis during neuronal differentiation.

**Apoptosis**

**PS4-036**

Histone acetylation status in NIH 3T3 cells after treatment with various inducers of apoptosis

H. Talasz and B. Puschendorf
Institute of Medical Chemistry and Biochemistry, Austria

Background: Histone hyperacetylation, caused by histone deacetylase inhibitors, is discussed to play a role in generating apoptosis. The precise mechanism, however, by which the inhibitors induce apoptotic cell death remains obscure. In our study we compared the effect of various apoptosis inducers on histone acetylation pattern.

Methods: NIH 3T3 cells were treated with the deacetylase inhibitors butyrate and trichostatin A, or with tumor necrosis factor (TNF) alpha. The acetylation pattern and the 14C-acetate incorporation were assessed using one- or two-dimensional acid urea gel electrophoresis.

Results: In NIH 3T3 cells, after 8 h of incubation the treatment with the deacetylase inhibitors butyrate (10 mM) or trichostatin A (1 µM) clearly led to hyperacetylated core histones, however, without inducing apoptotic cell death. Using the combination of butyrate (or trichostatin A) with TNP-Falp, 50% of the cells showed apoptosis with decreased levels of hyperacetylation as compared to butyrate or trichostatin A treatment alone. Eighty percent of the cells showed signs of apoptotic cell death when the combination of cycloheximide and TNP-Falp exhibited deacetylated core histones and reduced acetate incorporation as compared to control cells. Histone H2A.X, a marker of DNA damage, showed reduced acetylation pattern and acetate incorporation whereas hyperphosphorylated H2A.X forms increased.

Conclusions: Our results show that depending on the inducer either hyperacetylation or hypoaetylation of histones may be necessary but not sufficient for induction of apoptosis.

**PS4-037**

TAB2 a TAK-1 binding protein, regulates p73b function through protein–protein interaction

T. S. Kim, K. C. Kim, S. Ryu and K. H. Choi
Department of Biological, Chung-Ang University, Seoul, South Korea

A nuclear protein (p73) that is similar in structure and function to p53. Notably, the C-terminal region of p73 has a regulatory function, through interactions with a positive or negative regulator. In this study, we use the yeast two-hybrid technique to identify a p73 binding protein, TAB2. TAB2 is TGF-beta-associated kinase (TAK1) binding protein 2, and is known to be an adaptor molecule in TAK1 regulated MAP kinase pathways. It was confirmed that TAB2 binds full-length p73b, both in vitro and in vivo. This association is mediated via C-terminal regions of TAB2 and p73b. Overexpression of TAB2 was found to increase the transcriptional activity of p73b in a dose-dependent manner. In addition, the cell death function of p73b was increased by TAB2. These results imply that TAB2 modulates p73b function by direct binding.

**PS4-038**

The role of ternary complex factors (TCFs) in neuronal survival

I. Aksan, E. R. Vickers and A. D. Sharrocks
University of Manchester, UK

Ternary complex factors are a subclass of the ETS domain transcription factors, best characterized for their role in immediate early gene (IEG) regulation, most notably c-fos. They are activated by MAPK pathway in response to a variety of stimuli, and form ternary complexes with Serum Response Factor (SRF) on serum response elements (SRE) of target promoters. Their association with IEGs suggests a role in proliferation. By stable expression of a dominantly acting repressor form of Elk-1 in HEK293 and PC12 cells, we show that blocking expression of TCF-regulated genes triggers apoptosis irrespective of cell type, in an SRF-dependent manner. We further present evidence that Elk-1 has a role in neuronal differentiation in PC12 model system. We propose that TCFs act as key factors in regulating cell survival and/or protecting from apoptosis during neuronal differentiation.

**PS4-039**

Cell death in response to etoposide and to indoloquinolines proceeds by different mechanisms not involving CD95 receptor

E. Marcinkowska and R. Humeniuk
Institute of Immunology and Experimental Therapy, Polish Academy of Science, Weigl St. 12, 53–114 Wroclaw, Poland

Natural indoloquinolines and their synthetic analogs were supposed to induce cell death by inhibition of topoisomerase II, similarly to etoposide, which is a topoisomerase II poison. We have shown in a model of different leukemic cell lines with variously expressed topoisomerase II, that it is very unlikely that inhibition of this enzyme mediates cytotoxic effect of indoloquinolines. In five different leukemic cell lines we show four different patterns of sensitivity to etoposide and to necrotopo9lepine (belonging to the group of indoloquinolines). As far as sensitivity of the cells to etoposide is related to the cellular content of topoisomerase II, such link does not exist for necrotopo9lepine. In sensitive cells etoposide induces rapid process of apoptosis, what also arguments against involvement of CD95 receptor in the etoposide-induced cell death. Altogether we show that necrotopo9lepine in contrary to etoposide has different than topoisomerase II cellular target, and that both anticancer drugs induce cell death by mechanism most probably not involving CD95 receptor.
Myocardial ischemia induces apoptosis in mitochondrial permeability transition-dependent manner

A. Jekabsons1, V. Borutaite1,2, R. Morkuniene1 and G. C. Brown2
1Institute for Biomedical Research, Kaunas University of Medicine, Kaunas, Lithuania, 2Biochemistry Department, University of Cambridge, Cambridge, UK

Ischemia and reperfusion of heart causes contractile dysfunction, cell death, and is a major cause of human mortality, but the molecular mechanisms are unclear. We show that ischemia alone (without reperfusion) is sufficient to induce apoptosis and mitochondrial dysfunction, and we have investigated the mechanism responsible. After rat heart perfusion on Langendorf system, 30 and 60 min stop-flow ischemia was induced. Functions of isolated mitochondria were determined by simultaneously measuring respiration rate and membrane potential, and from respiratory activity of skinned muscle fibers. Cytochrome c content in mitochondria and fibers was determined spectrophotometrically, and in cytosolic fractions by Western blotting. Caspase-3-like activity was determined spectrophotometrically, and DNA strand breaks – immunocytochemically. Thirty and 60 min ischemia induced: (a) progressive release of cytochrome c from mitochondria to cytosol (b) inhibition of the mitochondrial respiratory functions (c) activation of caspase-3-like protease activity (d) DNA strand breaks. Fifteen minutes preperfusion with cyclosporin A, an inhibitor of mitochondrial permeability transition, largely prevented all these ischemic changes. Preperfusion with FK506, an inhibitor of calcineurin, caused no protection. Preperfusion with DEVd-CHO, an inhibitor of caspase-3-like proteases, completely prevented ischemia-induced DNA strand breaks but only partially blocked cytochrome c release and mitochondrial respiratory inhibition. We conclude that ischemia-induced mitochondrial permeability transition causes release of cytochrome c, which then activates the caspases that execute apoptosis and feedback to cause further cytochrome c release. The mitochondrial permeability transition-induced cytochrome c release is also largely responsible for the ischemic respiratory inhibition, which might contribute to contractile dysfunction or necrosis at reperfusion.

Identification of Ask-1 as an E2F1 transcriptional target gene

Z. Kherrouche1, Y. De Launois1,2 and D. Monte4
1FREE2537, Institut de Biologie de Lille/Institut Pasteur de Lille, 1 rue Calmette 59000 LILLE, France, 2Laboratoire de Vinologie Moleculaire, Faculté de Médecine, Universite Libre de Bruxelles, CP614, 508 Route de Lennik, 1070 Brussels, Belgium

E2F1 is a key regulator of cell cycle progression that also displays a p53 dependent or independent apoptotic function in response to perturbations of normal cell proliferation. In order to identify E2F1 target genes implicated in such process, we performed a Representational Difference Analysis of cDNA between RNA from p53 deficient Saos-2 cells infected by recombinant adenovirus expressing E2F1 or Green Fluorescent Protein as a control. We show here that E2F1 regulates the RNA and protein expression levels of the apoptosis signal regulating kinase 1 (Ask-1), a homologous protein is also present in Zebra fish, indicating that NUCKS is a vertebrate specific protein. We found that 10 min of heart perfusion with GSNO substantially inhibits mitochondrial respiratory chain, induces cytochrome c release from mitochondria and causes about three-fold increase in caspase-3-like protease activity, GSNO-affected mitochondrial respiration was restored after addition of exogenous cytochrome c to the incubation medium suggesting that mitochondrial inactivation was mainly due to the loss of this protein. The effects of GSNO was completely prevented by adding cyclosporin A, a selective inhibitor of mitochondrial permeability transition, to the perfusion buffer: after perfusion of hearts together with GSNO and cyclosporin A there was no inhibition of mitochondrial function, neither decrease in mitochondrial cytochrome c content nor increase caspase activity. GSNO also induced cyclosporin A-sensitive nuclear DNA fragmentation in cardiomyocytes of the perfused hearts. In conclusion, GSNO may induce cardiomyocyte apoptosis mediated by mitochondrial permeability transition.

The NUCKS gene is located on human chromosome 1: possible function of NUCKS in apoptosis?

I. Haga and A. C. Ostvold
Institute of Medical Biochemistry, Neurochem. Section, University of Oslo, Oslo, Norway

The novel gene NUCKS is located on chromosome 1q32.1 and consists of seven exons and six introns. The gene lacks a TATA box but contains two INR elements, two GC boxes and one consensus binding site for E2F-2. NUCKS is expressed in all human adult and fetal tissues investigated and has all the features of being a housekeeping gene. Southern Zoo blot analyzes show that NUCKS is present in a number of mammals. A homologous protein is also present in Zebrafish and Xenopus laevis but not in Drosophila and yeast, suggesting that NUCKS is a vertebrate specific gene. Although the NUCKS gene is constitutively expressed in all cell types, the protein is highly up regulated in apoptotic cells.

Heart perfusion with NO donor S-nitrosogluthathione activates caspases via mitochondrial pathway

Z. Dapkus1, A. Jekabsons2 and V. Borutaite1,2
1Department of Biology, Faculty of Natural Sciences, Vytautas Magnus University, Kaunas, Lithuania, 2Institute for Biomedical Research, Kaunas University of Medicine, Kaunas, Lithuania, 3Department of Biochemistry, University of Cambridge, Cambridge, UK

Increased NO may induce cytochrome c release and caspase activation, but the mechanism is not clear. Wistar rat hearts were used in experiments. Heart perfusion was performed on Langendorf system using Krebs-Henseleit solution supplemented with 1 mM nitrosating NO donor S-nitrosothioglutathione (GSNO) or GSNO together with 0.1 μM cyclosporin A. For control, hearts were perfused without specific additions. Mitochondrial and cytosolic fractions from perfused hearts were isolated by differential centrifugation. Mitochondrial activity was investigated by measuring respiratory rate and membrane potential simultaneously. Citochromes c + c1 and in mitochondria, and caspase-3-like protease activity in cytosolic fractions were determined spectrophotometrically. Nuclear DNA double strand breaks in tissue sections from GSNO-treated hearts were stained immunocytochemically. We found that 10 min of heart perfusion with GSNO substantially inhibits mitochondrial respiratory chain, induces cytochrome c release from mitochondria and causes about three-fold increase in caspase-3-like protease activity. GSNO-affected mitochondrial respiration was restored after addition of exogenous cytochrome c to the incubation medium suggesting that mitochondrial inactivation was mainly due to the loss of this protein. The effects of GSNO was completely prevented by adding cyclosporin A, a selective inhibitor of mitochondrial permeability transition, to the perfusion buffer: after perfusion of hearts together with GSNO and cyclosporin A there was no inhibition of mitochondrial function, neither decrease in mitochondrial cytochrome c content nor increase caspase activity. GSNO also induced cyclosporin A-sensitive nuclear DNA fragmentation in cardiomyocytes of the perfused hearts. In conclusion, GSNO may induce cardiomyocyte apoptosis mediated by mitochondrial permeability transition.
the specific proteolysis of pro-caspases for three hours after a 140-J/m² irradiation. The time course of caspases processing showed an activation of caspase 3, after 1 h and an activation of caspase 8 after 2 h from irradiation. Furthermore, we confirmed caspase 3 activation by analyzing the degradation of the inhibitor of caspase-activated-Dnase (DFF-45), that was shown to occur after 2 h from irradiation. The fast caspase 3 activation seems to point out that the UV-C apoptotic induction in HL-60 cells, is mainly due to cytoplasmic events and does not involve variation on gene expression induced by DNA damage. According to the time course of caspases activation, pro-caspase 8 is processed after caspase 3 and most probably through a Fox independent mechanism, since HL-60 was shown to be resistant to FasL, or anti-Fas-induced apoptosis. Irradiation with ultraviolet light at 254 nm (UV-C) is a powerful inducer of DNA damage. It has been shown that UV-C irradiation is able to induce apoptosis in some cellular systems, although the mechanism involved in this event is still unclear. We explored the ability of UV-C radiation to induce apoptosis in HL-60 cell line with the rationale of dose range condition. After 24 h from irradiation, we observed a dose-dependent increase of the number of apoptotic cells and DNA ladder fragmentation. The DNA fragmentation was detectable after 70 J/m² irradiation and reached its maximum at 280 J/m². To demonstrate that DNA fragmentation was due to the apoptotic induction, we analyzed caspase 3 and caspase 8 activation. We followed the specific proteolysis of pro-caspases for three hours after a 140-J/m² irradiation. The time course of caspases processing showed an activation of caspase 3, after 1 h and an activation of caspase 8 after 2 h from irradiation. Furthermore, we confirmed caspase 3 activation by analyzing the degradation of the inhibitor of caspase-activated-Dnase (DFF-45), that was shown to occur after 2 h from irradiation. The fast caspase 3 activation seems to point out that the UV-C apoptotic induction in HL-60 cells, is mainly due to cytoplasmic events and does not involve variation on gene expression induced by DNA damage. According to the time course of caspases activation, pro-caspase 8 is processed after caspase 3 and most probably through a Fox independent mechanism, since HL-60 was shown to be resistant to FasL, or anti-Fas-induced apoptosis.

**PS4-047**

**Reaction mechanism of caspases: new insights from theoretical calculations**

M. Sulpiçi1, U. Rothlisberger1, A. Cattaneo2 and P. Carloni3
1Laboratory Inorg. Chem. ETHZ, Hoengerberg Zurich, Switzerland.
3SISSA/ISSAS, via Beirat 2-4, 34014 Trieste, Italy.

Caspases are fundamental targets for pharmaceutical interventions in a variety of diseases involving deregulated apoptosis. Here, we present a QM/MM [1], and Car and Parrinello [2] study of key steps of the enzymatic reaction for a representative member of this family, caspase-3 (C-3). In particular, C-3 is a key enzyme in neurodegeneration of the Alzheimer’s disease [3, 4]. The hydrolysis of the acyl-enzyme complex is described at the density functional (BLYP) level of theory while the protein frame and solvent is treated by the GROMO8-3 force field. These calculations show that the attack of the hydrolytic water molecule implies an activation free energy of ca. 20 kcal/mol in good agreement with experimental data, and leads to a previously unrecognized gem-diol intermediate that can readily (free energy barrier ~5 kcal/mol) evolve to the enzyme products. Our findings assist in elucidating the striking difference in catalytic activity between caspases and other structurally well-characterized cysteine proteases (papains and catalases) and may help in the design of novel transition-state analog inhibitors.

**References:**


**PS4-048**

**Caspase-3 activation in AMPA receptor-mediated neurotoxicity in cultured hippocampal neurons: role of NO**

E. C. Leal, I. M. Araujo, A. F. Ambrosio, A. P. Carvalho and C. M. Carvalho
Center for Neuroscience and Cell Biology, Department of Cell Biology, University of Coimbra, Coimbra, Portugal.

In this study, we investigated a possible correlation between AMPA receptor-mediated neurotoxicity, in nondesensitizing conditions, and NOS activation and NO production in cultured hippocampal neurons. Short-term exposures of the cells to KA (5 and 15 min) in the presence of cycloheximide (CTZ), decreased the MTT reduction to 77.1 ± 1.7 and 70.8 ± 4.0% of the control, respectively. Both NBQX and D-AP-5 abolished the neurotoxic effect of KA + CTZ, suggesting that both AMPA and NMDA receptors are involved, either directly or indirectly, in the neurotoxic effect of KA exposure in nondesensitizing conditions. The addition of NOS inhibitors (L-NAME or 7-NI) partially or totally prevented the toxicity induced by KA + CTZ. Along with a decreased cell viability, we observed that exposure to KA + CTZ also lead to decreased intracellular ATP levels and increased caspase-3 like activity. Accordingly, NBQX, D-AP-5 and NOS inhibitors prevented (partially or totally) the ATP depletion and the increase caspase-3 like activity. We also measured cGMP levels as an indirect assay for NO production. KA + CTZ exposure for 15 min significantly increased cGMP levels, which was prevented by L-NAME and ODQ. Overall, these results show that NO and caspase-3 like enzymes plays a role in the neurotoxicity caused by the activation of AMPA receptors in cultured hippocampal neurons. Supported by FCT, Portugal: Grants SFRH/BDE/3120/2000, SFRH/BPD/858/2000, Project POCTI/1999/NES/35875.

**PS4-049**

**Atypical protein kinase Cota as an antiapoptotic effector of c-Abl following DNA damage**

F. Ueberall1, M. Jenny1, F. Hochholdinger1, H. Grunicke1 and M. Spitaler2
1Institute of Medical Chemistry and Biochemistry, University of Innsbruck, 2Lymphocyte Activation Laboratory, Cancer Research UK, 44 Lincoln’s Inn Fields, London WC2A 3PX, UK.

DNA-damaging agents initiate a signalling cascade resulting either in cell cycle arrest and DNA repair or apoptotic cell death. Immediate response to DNA strand breaks involves the nonreceptor tyrosine kinase c-Abl, which is directly activated by damage-recognizing serine/threonine kinases like Ataxia-Telangiectasia Mutated (ATM) and is essential for both the antiproliferative and the apoptotic responses. However, constitutively active forms of c-Abl like v-Abl or Bcr-Abl can act as oncogenes, over- coming antiproliferative and proapoptotic effects. This could indicate an as yet unknown physiological effect of c-Abl. One central effector of the Bcr-Abl oncogene, necessary for the induction of the drug-resistant phenotype, is the atypical protein kinase C PKCota. As atypical PKCs are involved in a number of mitogenic and antiapoptotic signals of both tumor and normal cells, we questioned whether PKCota can also mediate such an antiapoptotic effect of cellular Abl. Here, we show that c-Abl interacts with PKCota both in vivo in COS cells and prostate carcinoma PC-3 cells, as well as in vitro upon incubation with recombinant PKCota. Elevated c-Abl activity lead to activation of PKCota by tyrosine phosphorylation in the catalytic domain, which can also be achieved by the DNA damaging agent camptothecin. Furthermore, by transient transfection of active or dominant negative PKCota constructs we could show that PKCota activity correlates with resistance against DNA damage. This shows that PKCota is a direct, antiapoptotic effector of c-Abl activated by DNA damage, an effect that could help to adjust the cellular response to the gravity damage.

**PS4-050**

**The Tibetan herbal remedy PADMA28 fights tumor cell growth in cell culture**

F. Ueberall, M. Jenny and W. Schweiger
Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Austria

There is growing public interest in natural products for confirmed diseases and, inspired by native folk medicine, national programs are running worldwide for large-scale screening of novel antitumour agents. Here, we show that the application of PADMA28, a classical Tibetan herbal formula, to human T-cell derived lymphatic leukemia cells (CEM-C7HE2), deficient in functional p53 and p16, leads to a growth inhibitory effect followed by the induction of programmed cell death (PCD). PADMA28-induced PCD was determined by FACS analysis, caspase-3-mediated degradation of poly(ADP-ribose) polymerase, intranucleosomal degradation of DNA, as well as single cell images of apoptotic body formation. Additionally, our investigations revealed that inhibition of atypical protein kinase C iota markedly enhances PADMA28-induced apoptotic body formation, indicating a synergistic effect of herbal components with putative atypical PKC inhibition in overwriting survival functions of tumor cells. This conclusion is further reinforced by the genetic ablation of aPKCota which further sensitizes the cells against PADMA28-induced PCD. With all the cautions called for in drawing conclusions from cell culture experiments, regular consumption of the Tibetan herbal remedy PADMA28 could be a synergistic therapy concept against acquired capabilities of cancer cells.

**PS4-051**

**Signaling events of Newcastle disease virus induced apoptosis in PC12 cells**

Z. S. Fabian1, K. Katalin1, J. Szerebenyi1 and L. K. Csátry2
1Department of Medical Biology, Faculty of Medicine, Pécs University, Pécs, Hungary, 2United Cancer Research Institute, Ft. Lauderdale, FL, USA

**Introduction:** Newcastle disease virus (NDV), a member of the group of avian paramyxoviruses, causes serious infections in birds, but is virtually nonpathogenic in humans. Recently, an attenuated NDV vaccine (MTH-68/II) was developed and found to cause significant regression of various human tumors in clinical trials. The oncogenic action of MTH-68/II, however, is still poorly understood. Since wild-type NDV is able to cause apoptotic cell death in a number of cultured avian cell types, programmed cell death may also be the oncolytic mechanism of MTH-68/II.
Enzyme engineering

PS4-052

Folding of native and mutant maize beta-glucosidase in E. coli is facilitated by thioredoxin fusion

J. Zoubal, M. Slany, and B. Brzobohaty
1Department of Functional Genomics and Proteomics, Faculty of Science, Masaryk University, Kołarska 2, CZ-61137 Brno, Czech Republic
2Department of Agricultural Sciences, Prague, Czech Republic

Expression in E. coli and biochemical characteristics of his-patch(hp)-thioredoxin fused to wild type and mutant maize beta-glucosidase Zm-p60.1 were analyzed. Earlier, when expressed as His-tag fusion proteins, 20% of the wild type enzyme and minor fractions of C479A/R/D mutants accumulated in the soluble, enzymatically active, dimer form, while C52A/D were found exclusively in inclusion bodies. A minor fraction of C52R was found as soluble protein, however, the mutant remained a monomer. When expressed as hp-thioredoxin fusion proteins, 80% of the wild type enzyme and large fractions of C52A/R/D mutants were found to be soluble. Moreover, all the proteins formed native homodimers possessing enzyme activity. Thus, the hp-thioredoxin facilitates folding of the fusion partner into a tertiary structure that accumulates as a soluble protein. We also demonstrate that the hp-thioredoxin part of the fusion protein can also facilitate folding into the correct tertiary structure that is competent to form native quaternary structure. Both His-tag and hp-thioredoxin fusions with C479A/R/D accumulated as correctly folded, catalytically active homodimers. Comparison of the kinetic parameters of the two types of fusion revealed that the hp-thioredoxin does not affect the catalytic properties of either the wild type or the mutants. Apparently, the hp-thioredoxin does not influence biological activity of the fusion partner upon completion of the folding process. This is crucial for subsequent analysis of structure-function relationships in the fusion proteins. Supported by grants 203/02/0865, Z504920 and MSM143100008.

PS4-054

Synthesis of new bacterial biodegradable plastics with broad applications

J. Luengo1, E. Olivera1, G. Garcia1, N. Naharro2 and A. Sandoval1
1Department Bioquímica y Biología Molecular, Universidad de León, 2Department Sanidad Animal, Universidad de León

When cultured in chemically defined media containing aromatic or aliphatic acids (C6 to C12) as carbon sources, Pseudomonas putida U accumulates several polymers (poly 3-hydroxy[phenyl]alkanoates) as reserve materials. Genetic analysis has identified a three-gene operon that encodes two polymerases and a dehalogenase which are responsible for the biosynthesis and degradation of these polymers. Further studies allowed the identification of other proteins (phasins), involved in the supramacromolecular organization of these polymers. The mutation or deletion of some of the genes belonging to these operons or those involved in the beta-oxidation pathway, elicits, in some cases, a strong intracellular accumulation of unusual homo- and copolymers that alter the morphology of these bacteria (more than 90% of the cytoplasm is occupied by these macromolecules). These mutants synthesize different polymers (other than those produced by the wild-type) with regard to both monomer size and relative percentage. Biotechnological studies revealed that these polymers, with plastic properties, as well as some of their derivatives, have important medical or pharmaceutical applications (antitumoral, analgesic, radioprotectors, chemo preventive or antihelminthic). In summary, these mutants could be used for the industrial production of a huge number of biodegradable polysteres with different characteristics and applications, opening up a plethora of potential biotechnological uses.

PS4-055

Investigation of physico-chemical and biological properties of L-lysine alpha-oxidase conjugates with antibodies and peroxidase

N. V. Gogichaeva, E. V. Lukasheva and T. T. Berezov
Russian Peoples Friendship University, Russia

L-Lysine alpha-oxidase was shown to be an active antitumor and antiviral enzyme. The cytotoxic effect was established against several human cell lines in vitro, anti-inflammatory and antitumorous activity were observed in vivo experiments. The attachment of biologically active substance to the monoclonal antibodies provides their transport to definite target tissues. In the present work, we demonstrate the elaboration of the methods for L-lysine alpha-oxidase conjugation with peroxidase and monoclonal antibodies and the investigation of the conjugates physico-chemical properties (Kv, pH-stability, thermal resistance) and cytotoxic activity. It was shown that the inhibition of the Yurkat lymphoma and Ca Ov. cell lines growth was proportional to the concentrations of both native enzyme L-lysine alpha-oxidase and its conjugates with monoclonal antibodies. The data obtained showed that the native form of L-lysine alpha-oxidase was more effective cytotoxic inhibitor than conjugated form of enzyme. The conjugates had reduced thermal stability in comparison with native L-lysine alpha-oxidase: Kv was increased, it means that the chemical affinity to L-lysine in conjugates was diminished, the pH-stability did not change.

PS4-055

Liver gamma-butyrobetaine hydroxylase activity in non-pregnant (dry), pregnant and lactating rabbit

A. Coskun and M. Dogan
1Department of Clinical Biochemistry, Kirikkale State Hospital, Kirikkale, Turkey, 2Department of Dairy Science, Safran Crikicioglu College, Erzurum University, Karsiyar, Turkey

Gamma-butyrobetaine hydroxylase (also called gamma-butyrobetaine, 2-oxoglutarate dioxygenase (EC 1.14.11.1)) is a key enzyme in carnitine biosynthesis and catalyzes the hydroxylation of gamma-butyrobetaine to form L-carnitine. Carnitine concentrations in the plasma of pregnant women was reported to decrease as gestation proceeds. In contrast to plasma, only limited information is available about carnitine metabolism in the liver during pregnancy and lactation periods. The aim of this study was to investigate liver gamma-butyrobetaine hydroxylase activity during pregnancy and
PS4-057
Structures of fungal laccases with a full complement of coppers: substrate specificity and reactivity
K. Piontek1, T. Choinowski1, M. Antorini1, I. Herpoel-Gimbert2, M. Asther2 and J. C. Sigismond2
1Swiss Federal Institute of Technology, Zurich, Switzerland. 2INRA. Universités de Provence et de la Mediterranee, Marseille, France
Laccases belong to the family of blue multicopper oxidases, which catalyse four one-electron oxidations of reducing substrate with the concomitant four-electron reduction of dioxygen to water. These enzymes oxidise substrates such as phenols and aromatic amines. They are found in plants, bacteria, insects, and fungi. In trees laccases are involved in lignin synthesis, while fungal laccases play a role in lignin degradation. Previously, only a crystal structure of a laccase in an inactive Cu-depleted form has been reported, where the putative type-2C4 is completely absent. We report now on the first crystal structures of laccases, which contain all four coppers and which allow a first detailed view into the three coppers of the type-2/type-3 clusters that of ascorbate oxidase and of mammalian plasma ceruloplasmin, suggesting a common reaction mechanism for the copper oxidation and the oxygen reduction. Besides a structure of the oxidised form, structures of substrate bound, reduced laccases have been determined. The substrate binds in a small cavity close to the type-1 copper. By comparison with ascrobate oxidase the substrate specificities of the two enzymes can be explained. Contrary to most blue copper proteins the type-1 copper in fungal laccases has no axial ligand and is only three-fold coordinated. Previously, the lack of an axial ligand has been attributed to a modest elevation of the redox potential. Based on structural data and sequence comparisons, a mechanism is proposed by which laccases can tune their redox potential by about 200 mV.

PS4-058
PG and PL activity studies on enzymes with textile applications
M. Onos, L. Del Valle, M. Calafell and P. Garriga
ETSEIT-UPC, Chemical Engineering, Terrassa, Spain
Cotton fibers are protected from environmental factors by their cuticle. Waxes are the main components responsible for the nonabsorbent features of crude cotton. Pectins are acidic polysaccharides with about 85% of methylated groups. Removal of these substances from the fiber is required for textile applications in a process called scouring. The main objective of our work is to optimize the process of enzymatic scouring. Two commercial pectinases and four noncommercial laboratory-obtained pectinases are analyzed. These enzymes differ in their activity and pH. One of the commercial enzymes, of acidic optimal pH, is of common use in the preparation of juices and gelatines in food applications. The commercial enzyme of basic optimal pH is a bacterial pectinase especially produced for the textile biocouring process. Several techniques have been used aiming at determining the effectiveness of the different enzyme treatment. We have also measured protein patterns in enzymatic preparations by SDS-PAGE electrophoresis and quantified the amount of protein with the Bradford assay. The activity of the enzymes has also been analyzed by peptide inhibition of several loops surrounding the active site of the TEM-1 beta-lactamase was evaluated. Random sequences were inserted in permisive sites to generate first generation libraries and the active fractions of each library were recovered by invivo selection (ampicillin resistance). Combination of active libraries afforded combinatorial libraries with multiple random inserts and with a high proportion of active clones. From these libraries, active enzymes binding with high affinities to various monoclonal antibodies were selected by phage display. The activity of these enzymes is markedly affected upon antibody binding. Furthermore, beta-lactamase clones binding to horse spleen ferritin, streptavidin and beta-galactosidase could also be isolated. Affinity maturation of a clone binding to ferritin allowed obtaining chimeric enzymes with affinities comprised between 10 and 20 nM (Kd) for the protein. Contrarily to what was observed for beta-lactamases issued from selected on antibodies, enzyme complexation induced only a modest effect on enzyme activity. These chimeric enzymes could prove useful in replacement of enzyme-conjugated antibodies in enzyme-linked immunosorbant assay (ELISA) or in a new type of homogeneous assays when large activity effects are observed. These results also constitute an example of successful engineering of allosteric sites starting from a nonregulated enzyme.

PS4-059
Kinetic and structural characterization of two active forms of aspartic protease from murine intracisternal A-type particles
M. Sveč1, K. Strisovský2 and J. Konvalinka2
1Department of Theoretical Chemistry; 2Department of Biochemistry, Flemingovo Namesti 2, 16610 Praha 6, Czech Republic
Murine intracisternal A-type particles (IAPs) are endogenous retroviruses. The IAP particles assemble and bud at the membranes of the endoplasmic reticulum where they accumulate as immature particles consisting exclusively of uncleaved polypeptides. Recent evidence has indicated that the lack of proteolytic processing is not due to a defective viral protease but rather is caused by the site of particle formation and can be rescued by artificial redirection of the polypeptide to the plasma membrane. The recombinant protease of murine intracisternal A-type particle 14 (MIA14 PR) undergoes N- and C-terminal autoprocessing at defined sites and is sequentially and functionally related to the B- and D-type retrovirus proteases. An unusual feature of these proteases as opposed to the C-type retrovirus proteases is a 50 amino-acid C-terminal extension of unknown function. In this study, we aim to analyse the possible role of the C-terminal extension of the protease in regulation of polypeptide processing. We have cloned, expressed in E. coli and purified to homogeneity both the full-length MIA14 PR and its C-terminal truncated form. Both enzymes are active and have been used for invivo kinetic studies using peptide substrates and inhibitors. The C-terminal extension of MIA14 PR has been cloned, expressed in E. coli, purified and its effect on catalytic activity of both MIA14 PR constructs evaluated.

PS4-060
Molecular cloning of the gene coding for lactate-specific esterase and improvement of its thermo-stability from Pseudomonas sp. NT-40
M. Ishizuka1, M. Yoshida1, N. Karaki1, N. Nakagawa1, A. Kawagushi1, Y. Ogawa2 and K. Ushio2
1Department of Applied Chemistry, Chuo University, Tokyo, Japan, 2Nihon National College of Technology, Nihama, Ehime, Japan
Thermo-stable and high stereo-selective lipases and esterases have recently attracted much attention from viewpoints of industrial usage. In the course of our studies searching for efficient inducers for lipase, we have found that fatty alcohols act as ‘super-inducers’ for induction of thermostable lipase production by several Pseudomonas and other Pseudomonas-like bacteria including NT-40 [1]. Improvement of expression efficiency of lipase from several strains by fatty alcohols has been also reported [2]. In the course of analyzing the mechanism of lipase over-production, the gene of an esterase from Pseudomonas sp. NT-40 was isolated from the genomic library and sequenced. NT-40 esterase expressed mainly bound loosely to the cytoplasmic membrane of Escherichia coli on an equality with NT-40. Fatty alcohols were not so effective for the over-production of NT-40 esterase in E. coli. NT-40 esterase shows high homology among other lactone-specific esterases, and G-X-S-X-G consensus sequence is also conserved. Three mutant plasmids with the ability to produce the thermo-stable esterase were screened by the method of DNA shuffling with polymerase chain reaction. Lactones are widely distributed in nature, and have been synthesized from the corresponding omega-hydroxyfatty acids, need for specific lipases and esterases. It was reported that fatty alcohols as analogs of omega-hydroxyfatty acids (one of cutin monomers) can induce cutinase in a fungal system. A similar mechanism may be involved in bacterial lipase super-induction system.

References:

PS4-061
Orthogonal selections for engineering regulation sites on an enzyme scaffold
P. Soumilion, D. Legendre, P. Mathonnet and J. Fastrez
University Catholique De Louvain, Belgium
The combination of phage display and invivo selection allows generating chimeric beta-lactamases endowed with new specific binding properties directed to virtually any protein substrate. Initially, the insertion tolerance of several loops surrounding the active site of the TEM-1 beta-lactamase was evaluated. Random sequences were inserted in permisive sites to generate first generation libraries and the active fractions of each library were recovered by invivo selection (ampicillin resistance). Combination of active libraries afforded combinatorial libraries with multiple random inserts and with a high proportion of active clones. From these libraries, active enzymes binding with high affinities to various monoclonal antibodies were selected by phage display. The activity of these enzymes is markedly affected upon antibody binding. Furthermore, beta-lactamase clones binding to horse spleen ferritin, streptavidin and beta-galactosidase could also be isolated. Affinity maturation of a clone binding to ferritin allowed obtaining chimeric enzymes with affinities comprised between 10 and 20 nM (Kd) for the protein. Contrarily to what was observed for beta-lactamases issued from selected on antibodies, enzyme complexation induced only a modest effect on enzyme activity. These chimeric enzymes could prove useful in replacement of enzyme-conjugated antibodies in enzymelinked immunosorbant assay (ELISA) or in a new type of homogeneous assays when large activity effects are observed. These results also constitute an example of successful engineering of allosteric sites starting from a nonregulated enzyme.
PS4-062

Genes and enzymes required for the biosynthesis of the commercial bacterial exopolysaccharide gellan
Centro de Engenharia Biologica e Quimica, Instituto Superior Tecnico, 1049-001 Lisboa, Portugal

The commercial gelling agent, gellan, is an exopolysaccharide (EPS) produced by Sphingomonas paucimobilis ATCC 31461, composed of a repeat unit with D-glucose, L-rhamnose and D-glucuronic acid (2:1:1), with a glycerate and acetate substituents. Gellan biosynthesis starts with the intracellular formation of the nucleotide-sugar precursors, whose pathway was partially elucidated in our laboratory, with the identification and characterization of phosphoglucomutase, encoded by pgmG [1] and UDP-glucose-pyrophosphorylase, encoded by ugpG [2]. Gellan repeat unit is formed by sequential transfer of the sugars to an activated lipid carrier, by committed glycosyltransferases (GT), followed by gellan polymerization and export. The gellan cluster includes genes coding for enzymes involved in dTDP-L-rhamnose-synthesis, four GTs and proteins required for gellan polymerization, export and possibly for regulation of gel genes expression [2, 3]. The beta-1-4-glucuronosyltransferase, that catalyses the glucuronic acid linked to the polymerized gellan-glucosaccharide, is encoded by gelK [3], and gelC, gelD, and gelE encode proteins homologous to those involved in polysaccharide chain length regulation and export in several bacteria. Experimental evidences indicate that they are indeed required for gellan biosynthesis since deletion of gelC, gelD or gelE impairs EPS production. These and other results supporting the role of these proteins in gellan chain length regulation and export will be shown. The genetic engineering of the gellan pathway through the augmentation of the expression of gellan biosynthetic genes, either individually or coordinately, will also be presented and discussed.

References:

PS4-063

Phage display of a cofactor containing protein for enzyme evolution
S. W. Vetter and D. B. Goodin
The Scripps Research Institute, Department Molecular Biology, La Jolla, USA

The evolution of proteins with novel catalytic properties has been undertaken for a long time and is driven by the potential biotechnological application of these new enzymes, as well as by the desire to gain a better understanding of the relationship between protein structure and catalysis. The alteration of existing enzymes is achieved routinely using directed evolution. True ‘de novo’ enzymes have been created in the form of catalytic antibodies, taking advantage of the immune system and phage display technology for the evolution and selection of enzyme like antibodies. However, these methods have intrinsic limitations that restrict their use. We set out to create a ‘de novo’ enzyme (1) that (1) uses a simple, single domain protein framework (2) is not derived from an existing enzyme (3) catalyzes a chemical transformation involving two or more reactants. We chose a member of the lipocalin protein family to provide a barrel-like protein framework and the oxidation/oxygenation of small organic molecules as the targeted chemical transformation. We introduced mutations into the protein based both on rational design and by random mutagenesis. The proteins were expressed as phage display libraries and screened for catalytic activity. We present evidence that the phage displayed protein can acquire a heme cofactor during phage maturation and that limited catalytic peroxygenase activity can be measured in samples containing less than 100 femtomoles of catalyst.

PS4-064

The expression of the E. coli penicillin acylase (pac) gene mutated on the transcriptional and translational control regions
S. I. Ozturk, F. Gumusel and E. Bermeke
1 Tubitak-Research Institute for Genetic Engineering and Biotechnology, Gebze, Kocaeli, Turkey; 2 Department of Molecular Biology and Tubitak-Research Institute for Genetic Engineering and Biotechnology, Gebze Institute of Technology, Faculty of Sciences, Gebze, Kocaeli, Turkey.

Penicillin acylase (PA) hydrolyzing penicillin G, is an industrially important enzyme since one of its products (6-aminopenicillanic acid) is used as a raw material for the production of semisynthetic penicillins. The pac gene which is responsible for the synthesis of PA, has been cloned from E. coli and the active enzyme has been expressed constitutively (Gumusel et al. 2001). Although the pac gene was cloned downstream of lac promoter in the developed recombinant strain pac:pUC 19 – E. coli JM 109, the evidence suggested that the expression of the pac gene could be directed by its own promoter rather than the lac promoter. The aim of the present study, was first the determination of the promoter from which pac gene expression was originated and then improvement of the expression efficiency through the mutations introduced to the transcriptional and translational control regions of the gene. To introduce the mutations, the Site Directed Mutagenesis method based on Polymerase Chain Reaction has been used. The evidence indicated that the expression of the pac gene could be directed by pac and/or lac promoter. An increased gene expression and enzyme activity were obtained with the developed recombinant strain containing the promoter mutation and also modification in the space between Shine Dalgarno (SD) region and AUG start codon comparing to the recombinant strain carrying the wild-type gene.

PS4-065

Comparative study of the chemical mechanism of methionine sulfoxide reductases from classes A and B
A. Olry, M. Antoine, S. Azza and G. Branlant
Laboratoire MAEM UMR 7567 CNRS-UPHP Faculté des Sciences BP 239 Vauvenargues-les-Nancy, France

Methionine oxidation into methionine sulfoxide is known to be involved in many pathologies and to exert regulatory effects on the function of the modified proteins. The oxidation process can be reversed by a family of enzymes named methionine sulfoxide reductases classes A and B), which can be divided into two subclasses named MsrA and MsrB. MsrA and MsrB display (1) opposite stereoselectivity towards the R and S sulfoxide function; (2) no sequence identity and; (3) different three-dimensional structures. On the other hand, MsrSs share a common two-step chemical mechanism including (1) sulfoxide reduction through a nucleophilic attack of an essential Cys towards the sulfur atom of the sulfoxide leading to formation of a sulfenic acid intermediate on the Cys and release of 1 mol of methionine per mol of enzyme and; (2) regeneration of the oxidized enzyme by a thio-redox-dependent recycling process through thiodisulfide exchange(s). Biochemical and kinetics data will be presented which illustrate the nature of the limiting step and the role of the aminoacids which are involved in the catalytic mechanism and in the substrate specificity of both subclasses of Msr.

PS4-066

Functional properties of Escherichia coli penicillin G acylase stabilized by carboxymethylcellulose conjugation
F. Ozturk, M. Kazan and T. Erarslan
The Scientific and Technical Research Council of Turkey, Kocaeli, Turkey

Escherichia coli penicillin G acylase (PGA) was conjugated by carboxymethylcellulose (CMC) for the stabilization of enzyme against temperature and pH. Prior to conjugation CMC was activated to its 2,3-diacetaldehyde derivative by periodate oxidation and active derivative was covalently linked to enzyme molecule. The first order inactivation kinetics was observed for both native and CMC conjugated PGA during their prolonged incubations at 40–60 °C temperature and 4–9 pH intervals. Lower inactivation rate constants, higher half-life times and higher free energies of inactivation were obtained for CMC conjugated PGA at temperature and pH values studied. These results indicate the stabilization of enzyme by CMC conjugation. Highest stabilization was obtained as nearly four-fold at 40 °C and pH 8.0. CMC conjugation did not cause any change on the temperature and pH profiles of PGA, however, activation energy of PGA was decreased from 37.24 to 33.32 kJ/mol. CMC conjugation also caused the reduction of Kcat value of PGA from 3.23 to 2.16 mT Pen G. On the other hand, kcat/km values of CMC-conjugated enzyme is increased 1.44 and 2.16 fold, respectively. These results indicate the improvement of catalytic performance and the enzyme-transition state complementarities of PGA after CMC. The activation free energy for transition state of CMC-conjugated PGA was found to be higher than that of native enzyme. However, the free energy of substrate (penicillin G) binding to native PGA was found to be higher than that of conjugated PGA. CMC conjugated enzyme released higher free energy of transition state binding. Activation entropy value of enzyme is also changed after conjugation as an indicator of the alteration of the conformational structure of enzyme.

PS4-067

Determination of the catalytic properties of alkaline protease from newly isolated Bacillus sp.
A. A. Denici, D. Kazan and A. Erarslan
The Scientific and Technical Research Council of Turkey, Kocaeli, Turkey

The catalytic properties of extracellular alkaline protease produced by newly isolated alkaliphilic Bacillus sp. was studied. Microorganism was cultivated at 37 °C and
Design of the LTR-based DNA substrate for recombinant HIV-1 integrase

E. A. Semenova, N. M. Gashnikova and A. G. Pokrovsky
Institute of Molecular Biology, State Research Center of Virology and Biotechnology 'Vector', Kolosovo, Russia

Integration of human immunodeficiency virus (HIV) DNA into the human genome requires the virus-encoded integrase (IN), and therefore the IN is a suitable target for antiviral strategies. Discovery of retroviral integrase is facilitated by the knowledge of the HIV-1 integrase complex. The components of these complexes can then be identified by mass spectrometry. In the present work, the precursor form of cardosin A was expressed in E. coli as inclusion bodies, refolded and purified. Autoactivation of the precursor form was induced at low temperature and the proteolytic processing mechanism was characterized in detail at the molecular level. Recombinant cardosin A followed a processing pathway distinct from the natural enzyme. However, the activity and specificity of both forms are similar, suggesting that this expression and purification procedure can be used to further analyze the structure-function relationship of plant aspartic proteases.

Expression, autoactivation and characterization of recombinant cardosin A

P. M. Castanheira, E. M. Pires and C. J. Faria
1Department of Biochemistry, University of Coimbra, 2Centre for Neurosciences of Coimbra, Portugal

Cardosin A is an aspartic protease from the flowers of cardoon that is known for its milk-clotting activity and has been suggested to be involved in plant reproduction. The protease is synthesized as a single chain precursor and then converted into its mature form by removal of an internal saposin-like domain and an N-terminal prosegment. In the present work, the precursor form of cardosin A was expressed in E. coli as inclusion bodies, refolded and purified. Autoactivation of the precursor form was induced at low temperature and the proteolytic processing mechanism was characterized in detail at the molecular level. Recombinant cardosin A followed a processing pathway distinct from the natural enzyme. However, the activity and specificity of both forms are similar, suggesting that this expression and purification procedure can be used to further analyze the structure-function relationship of plant aspartic proteases.