ICGEB International Centre for Genetic Engineering and Biotechnology | Developing Knowledge

ICGEB Research Grants Programme

RESEARCH GRANTS COMPLETED IN 2014

CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2014

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PERU	Patricia SHEEN	Transplanted proteins as new vaccine carriers
RUSSIA	Sofia G. GEORGIEVA	The role of transcription factor SAYP in nuclear dependent transcription
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SLOVAKIA	Branislav KOVACECH	Tau truncation: the self-renewing propagator of neurofibrillary degeneration in Alzheimer's disease
SRI LANKA	Neil Darrel FERNANDOPULLE	Characterisation and transfer of Drought Responsive Elements Binding (DREB) genes to rice and evaluation of their effects on drought tolerance
SUDAN	Hiba Salah-Eldin MOHAMED	Genetic epidemiology of common cancers in East Africa
TUNISIA	Héla KALLEL	Development of a novel vectored vaccine against Hepatitis E using Adeno-Associated Virus expressing truncated HEV capsid protein
TURKEY	Ali Kemal TOPALOGLU	Identification of novel genes taking part in human puberty

ARGENTINA

Title: Human respiratory syncytial virus biochemistry: uncovering novel antiviral targets
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ICGEB Reference No.: CRP/ARG10-02
Abstract: Infection by Respiratory Syncytial Virus (RSV) is the major cause of lower respiratory diseases worldwide, where mostly infants, but also immune-deficient, immune-compromised and elderly patients are highly susceptible to the virus, mainly through pneumonia and broncheolitis. The incidence in compromised socioeconomic groups is much higher, globally affecting 64 million people, 160,000 of which die every year, where 99% of them occur in developing countries. Like influenza, SARS, and other respiratory viruses, RSV is highly contagious, stable on environmental surfaces, and can mutate very rapidly, hampering the development of effective vaccines and antivirals. It shares several basic gene products with the paramyxoviridae family related to replication, attachment and fusion, which are also common to a large number of viruses of different families. However, it features three unique and distinctive proteins, namely, (i) the phosphoprotein (P), the RNA polymerase cofactor; (ii) the M2-1 transcription antiterminator, present only in pneumovirus, and (iii) the non-structural protein NS1, a type I interferon antagonist. With the aim of understanding fundamental, either comparative or distinctive, biochemical mechanisms behind essential events of the virus life cycle and uncover antiviral targets, we tackled the characterisation of these proteins.
The P tetramer is highly stable with a modular unfolding coupled to dissociation. It has an unusually large hydrodynamic volume, which is persistent upon formation of a complex with M2-1. The M2-1 tetramer shares a strikingly similar stability to P (36.8 and 37.3 kcal/mol, respectively), but it stability and dissociation is highly dependent on pH. Both proteins interact with with a KD of 8 nM through a singular tetramer-tetramer interface. M2-1 bears an essential cys3-his1 zinc-binding motif that can be found in Sendai and Ebola viruses and some eukaryotic transcription factors. We found that removal of the zinc atom leads to an Apo-M2-1 monomer, with secondary structure and stability identical to the tetramer. Dissociation is highly increased at pH 5.0 strongly suggesting that zinc removal, and therefore dissociation, is governed by the protonation of the histidine residue, indicative of an independent folding module with its non-specific RNA binding activity unaffected. Thus, the role of zinc goes beyond stabilisation of local structure, finely tuning dissociation to a fully folded and binding competent monomer. Removal of zinc is equivalent to the disruption of the motif by mutation, only that the former is potentially reversible in the cellular context. This process could therefore be triggered by a natural chelator such as glutathione or thioneins, where reversibility, expressed by the re-uptake of the metal by the Apo-species, strongly suggests a modulatory role of pH in the participation of M2−1 in the assembly of the polymerase complex or in virion budding. In addition, quantitative assessment of the hierarchy of these interactions and their mechanisms contribute to the general understanding of RNA replication and transcription in Paramyxoviruses. In particular, the unique P:M2-1 interface present in RSV provides a valuable antiviral target for this worldwide spread human pathogen.
The biological relevance and the fact that NS1 is unique to hRSV among paramyxoviruses, with no homology within databases and very importantly, no structure available, prompted us to investigate its conformational stability, equilibria and folding. Temperature cooperatively induces conformational changes leading to soluble spherical oligomers (NS1SOs) with amyloid- like or repetitive ß-sheet structures. The onset of the thermal transition is 45 °C, and the oligomerisation rate is increased by 25-fold from 40 to 46 °C, within physiological range. Conformational stability analysed by chemical perturbation of the NS1 monomer shows a two- state, highly reversible and cooperative unfolding. However, two transitions were observed in the chemical denaturation of NS1SOs: the first corresponds to a conformational transition and dissociation of the oligomers to the native monomer, indicating a substantial energy barrier. The second transition (2.0 to 3.5 M denaturant) corresponds to full unfolding of the native NS1 monomer. Overall, the requirement of a conformational change prior to dissociation indicates that the conformation of the monomer in the NS1SOs is substantially different from that of the native monomer. In addition, different co-solvent perturbations converged on the formation of ß-sheet enriched soluble oligomeric species, with secondary structure resembling those obtained after mild temperature treatment. Thus, a unique protein without homologs, structure or mechanistic information may switch between monomers and oligomers in conditions compatible with the cellular environment and be potentially modulated by molecular crowding or compartmentalisation. NS1SOs may act as a reservoir for increased levels and impact on protein turnover. Rather different solvent conditions cause irreversible self-oligomerisation of NS1, leading to discrete stable and spherical species (NS1SOs). The convergence of these conditions, including a mild temperature change, suggest that N1SOs

may accumulate in cells, where multiple conformational equilibria could be related to NS1's reported low binding specificity. This constitutes a comprehensive characterisation of one of the principal virulence factors of hRSV, directly involved in immune evasion by the virus, a fact that hampers the development of effective vaccines. The conformational plasticity and heterogeneity of this important viral polypeptide constitutes the biochemical foundations for understanding NS1 interactions, cellular localisation and turnover. This will ultimately reveal the underlying molecular mechanisms related to hRSV evasion of interferon-mediated innate immunity.

Publications:

Esperante, S.A., Chemes, L.B., Sánchez, I.E., de Prat-Gay, G. The respiratory syncytial virus transcription antiterminator M(2-1) is a highly stable, zinc binding tetramer with strong pH-dependent dissociation and a monomeric unfolding intermediate. 2011. Biochemistry **50(40)**, 8529-8539

Esperante, S.A., Paris, G., de Prat-Gay, G. Modular unfolding and dissociation of the human respiratory syncytial virus phosphoprotein p and its interaction with the m(2-1) antiterminator: a singular tetramer-tetramer interface arrangement. 2012. Biochemistry **51(41)**, 8100-8110

Esperante, S.A., Noval, M.G., Altieri, T.A., de Oliveira, G.A., Silva, J.L., de Prat-Gay, G. Fine modulation of the respiratory syncytial virus M2-1 protein quaternary structure by reversible zinc removal from its Cys(3)-His(1) motif. 2013. Biochemistry **52(39)**, 6779-6789

Pretel, E., Camporeale, G., de Prat-Gay, G. The non-structural NS1 protein unique to respiratory syncytial virus: a two-state folding monomer in quasi-equilibrium with a stable spherical oligomer. 2013. PLoS One **8(9)**, e74338

BANGLADESH

Title: Rapid production of transgenic doubled haploid (DHs) plants with *p68* and *PDH47* genes for drought and/or salinity stress tolerance using Bangladeshi rice cultivars

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Abstract: The *in vitro* production of doubled haploids (DHs) through androgenesis (anther and microspore culture) is an efficient system for production of fully homozygous plants rapidly. Traditionally, plant breeders can achieve homozygosity by using self-fertilisation or backcrossing, a time consuming process. By this technique complete homozygous plants can be produced within a year as compared to the long inbreeding method, which might otherwise take 5-10 years. Significant advantage is that the system not only speeds up the advance to homozygosity, but also increases selection efficiency. Doubled haploid plants are genetically normal and phenotypically stable. Abiotic stresses including drought and high salinity stress results in adverse effects on the growth of plants and the productivity of crops, thus resulting in significant economic losses worldwide. Minimising these losses is a major concern for all countries, including Bangladesh and India. Under this project drought and salinity have been considered, as these are arguably the most important problem worldwide and thus also for Bangladesh.

We have identified the elite genotypes that showed good performance to gametic embryogenesis, and a protocol has been developed for developing drought and/or salinity stress tolerant transgenic rice cultivars using *p68* gene. Recently, we have started work also using the second gene of *PDH47*.

This project is a combination of *in vitro* androgenesis (anther and microspore culture) and *Agrobacterium*-mediated gene transfer for the production of drought and/or salinity tolerant rice cultivars. For anther culture of rice regeneration potentials of 25 rice cultivars were assessed on the basis of anther response, embryo induction, plantlet regeneration and production of green and albino plantlets. Embryoids were obtained from only 5 out of 20 rice cultivars on media containing specific amino acids and different combination of phytohormones. In this case, IR43 produced maximum embryos (16.13%) and green plantlets (11.88%) at the followed by BRRI dhan 33, IR54, Jaya and BR3 in SK3 medium. All the responding genotypes produced albinos in addition to the green plantlets. Under this study anther and microspore-derived embryoids are used for rapid development of fully homozygous plant. For efficient microspore culture, six microspore isolation procedures (P_1-P_6) from three major groups were used, e.g. squeezed of rice floret and excised anthers by glass rod, rice floret and excised anther by homogeniser, rice floret and excised anther by blender isolation techniques. Microspore isolation procedure by glass rod (P_1) was much easier and the simplest method. A minor number of embryoids (65% from 100 anthers) were recorded when rice floret was squeezed by glass rod, whereas with the same procedure but isolation from anthers showed a good embryo yield (123%). For control, around two to four fold less embryoid (28%) were recorded when anthers were directly cultured in the same induction medium (AMC). In these cases, all microspore isolation methods showed good performance on embryo yield and total plant regeneration. But higher frequency of embryoids yield (177%) and green plantlets (109%) were recorded when anthers were squeezed by homogeniser and albino plants reduced compared to control. It may be concluded that, for production of high yield embryos and reducing albinisims, microspore isolation procedure is very much important besides other factors of androgenetic study.

Under this study we have successfully standardised the anther and isolated microspore culture procedure, optimised media and other culture conditions. That is a very important part for the success of doubled haploid production. We have cloned the RNA helicase gene (DB 10) to transformation and other related vectors and successfully completed also rice transformation procedure with our limitations, also rice transgenic has been developed. Analyses of transgenic plants are under progress. The outcome of this CRP-ICGEB research grant has been helpful for the standardisation of androgenetic work, standardisation of transformation protocol and rapid development of drought and salinity stress tolerant DHs transgenics, which is very important to develop transgenic homozygous lines shortly to mitigate the challenges of climate change for sustainable agriculture.

Objectives:

(i) Develop highly efficient system of getting haploid plants via anther/microspore culture in elite rice genotypes;

(ii) Using microspores/anther derived embryos/callus to develop *Agrobacterium*-mediated transformation system;

(iii) Developing haploid plants overexpressing *p68* and *PDH47* genes and confirmation of the transgenic lines for haploid nature and integration of foreign gene and their expressions;
(iv) Diplodisation of haploid transgenic lines to get homozygosity;
(v) Testing of transgenic plants for stress tolerance.

Results Obtained:

In the present study, 20 rice varieties were tested for assessment of their androgenic response. Each genotype was cultured separately on five induction media. Out of 20 varieties, a good number of embryoids and regenerated plantlets were observed in five genotypes viz. in IR43, BRRI dhan33, IR54, Jaya and BR3, which were cultured on N_6, R2, SK3, He2, and MO19 media. Among the five responding genotypes, IR43 showed the best response on SK3 medium, where the frequency of embryo production was 50.63%, followed by BRRI dhan33, IR54, Jaya and BR3. In this study Z1 medium was also tested but there was no callus induction response. It was observed that on N_6, He2, MO19 and R2 media the androgenic response was very poor in comparison to SK3. From this finding it may be concluded that out of five media, SK3 was the best induction medium for anther culture response in rice. Spikes, pre-treated at 4-7°C for different durations, were cultured on SK3 medium. Highest embryo production was found in a 5-day (T_3) induction period and their percentage was 88.88, 77.17, 79.67, 78.88 and 73.50% in IR43, BRRI dhan33, IR54, Jaya and BR3, respectively. The efficiency of green plantlet regeneration was 24.25, 19.67, 20.17, 19.13 and 17.33%, respectively. In all treatments there was significant increase in embryo formation and plant yield in comparison to the control. In T_3 frequency of embryos and green plantlet regenerants was better compared to all other treatments including the control. For isolated microspore culture we found the embryo yield and its regeneration potentials from six microspore isoaltion methods, e.g. P_1 = squeezed of rice floret by glass rod, P_2 = squeezed of excised anthers by glass rod, P_3 = squeezed of rice floret by homogenizer, P_4 = squeezed of excised anther by homogeniser, P_5 = squeezed of rice floret by blender, P_6 = squeezed of excised anther by blender were considered for this study. Fully formed embryoids were observed within 4-5 weeks of culture initiation. It was observed that out of three major methods, microspore isolation procedure by glass rod (P_1) was much easier, simpler and less expensive because for that, sophisticated equipment is not required. However, a lower percentage of embryoids (65% from 100 anthers) were recorded by glass rod isolation procedure, where green plants were recorded (47%), and a less number of albino plants (8%) in highest percentage of embryoids (177%) were recorded in P_4 where anthers were squeezed by homogeniser (Polytron). Out of the mentioned microspore isolation procedure, the best performance found in P_4 where anther were squeezed by homogeniser and in this case excised anthers were used as explants. The efficiency of isolated microspore culture technique of rice has been studied by three major methods and successfully standarised suitable isolation techniques. It was observed that better embryo yields of microspores isolation by omogeniser showed to be best than glass rod and blender isolation. But on that case regeneration was minor and albino plants obtained were little higher that with the other two procedures of glass and polytron isolation. In this study homogeniser (polytron) and blending isolation technique gave a very high number of embryoids per 100 anthers. Albinism is a great problem mainly to cereals crops and for androgenetic study and it has no agronomical value. However, under this study we found that the microspore isolation by sterile glass rod and homogeniser showed quite better performance and reducing albino plants compared with control and other isolation procedures. For plant transformation the p68 (DB10) gene cloned into pCAMBIA 1301 vector and for that transformation LBA4404 *Agrobacterium* strain was used. For rice transformation the standard protocol by Hiei et al. (2004) was followed. The gene was cloned for vector control (VC, without gene) sense (C14) and antisense (C1) and transformed to *Indica* rice calli. For rice transformation we found greenish calli and plantlets. Genomic DNA of transformants with *p68* were extracted from the putatively transgenic plants and control untransformed plants using modified CTAB method and found that out of 17 plants 2 showed transgenics. T_0 plants already transferred to pots for further molecular analysis.

Results Unforeseen in the Original Project:

Under this study we have mentioned two genes, namely *p68* and *PDH47*. Cloning part of *PDH47* gene is completed but due to time factor the complete success with PDH47 gene has not been completed yet. Due to unavailability of ploidy analyser we have not been able to perform analysis of plants for ploidy levels. But we are going to karyotypic study of plants for ploidy levels confirmation. We transferred transgenic plants (T_0) to pots under the *p68* gene. Southern and Northern blot analysis, some bioassay analysis of transgenics are not fully completed yet. But two Ph.D. students are still involved in this project for their Ph.D. thesis and we hope that will be able to complete our work.

Publications:

Siddique, A.B., Ara, I., Islam, S.M., Tuteja, N. Effect of air desiccation and salt stress factors on *in vitro* regeneration of rice (*Oryza sativa* L.). 2014. Plant Signal. Behav. **9(12)**, e977209

Islam, S.M.S., Ara, I., Tuteja, N., Subramaniam, S. Efficient microspore isolation methods for high yield embryoids and regeneration in rice (*Oryza sativa* L.). 2013. WASET **7(12)**, 891-896

Khatun, R., Islam, S.M.S., Ara, I., Tuteja, N., Bari, M.A. Effect of cold pre-treatment and media for improving anther culture of rice (*Oryza sativa* L.) in Bangladesh. 2012. Indian J. Biotech. **11**, 458-463

BRAZIL

Title: Interaction studies of cadherin from *Helicoverpa zea* as a receptor for Bt toxin Cry1Ac
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Abstract: This work had two main purposes: (i) to study the amino acid residues involved in the interaction of Cry1A toxins with Lepidopteran cadherin receptors and (ii) to evaluate the involvement of Lepidopteran G proteins on binding with Cry1Ac toxins for insect-pest control. In order to achieve these goals, *in vitro* and *in silico* techniques were applied, including Phage Display, Fluorescence assays, Mass spectrometry, Surface Plasmonic Resonance and molecular modeling.

Objectives:

(i) To determine the regions on Cry1Ac where cadherin receptors are interacting with, identifying the main amino acid residues involved in the binding through *in vitro* and *in silico* assays;
(ii) To evaluate and confirm the interaction between Cry1Ac and gGP from *H. virescens* through *in vitro* assays;
(iii) To determine the regions on Cry1Ac where gGP is interacting with through *in silico* assays.

Results Obtained:

In this report, we identified the gamma region of a G-protein from *Heliothis virescens* (HvgGP) as a potential receptor for Cry1Ac. Hence, using *in silico* analyses, we determined the structure of HvgGP and its interaction region with Cry1Ac. The binding sites were confirmed through Phage Display assays, using Cry1Ac as template. Fluorescence analyses indicated that HvgGP interacts with Cry1Ac in a specific region. Although the mode of action through membrane pore formation was already confirmed by several *in vivo* and *in vitro* assays, the mechanism through inhibition/activation of signalling pathways by the interaction with G protein complexes is still not clear. Considering the importance of G proteins on the activation of several signaling pathways and the role of Cry toxins in the agribusiness, we also propose a new mechanism of action for Cry1Ac, using HvgGP as the binding protein. Moreover, fluorescence analyses of Cry1Ac fragments, earlier obtained through Phage Display, and a fragment of cadherin from *H. armigera* were evaluated in order to identify the binding site of the Cry toxin with the insect receptor. Similarly, based on reports throughout literature, we used homology modeling, protein docking and molecular dynamics to simulate the interaction between *Manduca sexta*'s cadherin receptor BT-R_1 and *Bacillus thuringiensis*' Cry1Aa, Cry1Ab and Cry1Ac toxins. With our results, we were able to infer the most important residues participating in hydrogen bonds during receptor-ligand interaction. To further investigate the true importance of each binding region and validate our model, we synthetised peptides corresponding to each of these regions and also their homologous sites in Cry1Ac and *H. armigera* cadherin receptor. Hence, through Dynamic light scattering (DLS) and Surface Plasmon Resonance experiments, it was possible to confirm the binding specificity and affinity of receptor-ligand peptides.

Results Unforeseen in the Original Project:

Studies on *Helicoverpa zea*, which was the initial target for the respective study, could not be performed because of a loss of the insect colonies occurred to our Institute, which led to a redirection of projects and experiments.
Out of all the experiments proposed for the project, the production of Cry1Ac mutants was replaced by *in silico* and *in vitro* analyses of the interaction between Cry1A toxins and *M. sexta* cadherin receptor. As both methodologies aimed at the identification of specific amino acid residues related to the binding of Cry toxins with cadherin receptors, the second strategy was applied due to the innovative results they could provide to the project. Competition binding assays and analyses of irreversible binding were performed using different techniques, such as fluorescence and SPR, as we considered them to be more efficient and reliable methods for the purposes of the this research.

Publications:

Pelegrini, P.B., Martins-de-Sá, D., Lucena, A., Xavier, M-A., Freitas, S.M., Grossi-de-Sá, M.F. Cry1Ac binds to insect g-subunit of G proteins. 2014. Nat. Struct. Mol. Biol. (being submitted)

BULGARIA

Title: Coordination of DNA synthesis and unwinding by replicative checkpoint
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ICGEB Contract No.: CRP/10/010
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Abstract: The process of DNA replication includes duplex unwinding, immediately followed by DNA synthesis. The DNA synthesis and unwinding of DNA are highly synchronised even when the synthesis is disturbed in damaged DNA regions, in replication slow zones, or as a result of insufficient nucleotide level. Several recent works suggest that S-phase checkpoint pathways regulate both replicative unwinding and DNA synthesis to synchronise the two processes in order to preserve genome stability. When replication forks stall in response to genomic insults, the S-phase checkpoint pathways inhibit late origin firing, delay S/M-phase cell cycle transition and stabilise the replisome to facilitate the re-establishment of fork progression after stress removal.

The exact mechanisms of synchronisation of the replicative DNA unwinding and synthesis is still vague. It is also unclear which proteins are involved in synchronisation of replicative unwinding and synthesis and what is their particular role in this process.

The aim of our work was to find out proteins that regulate replicative unwinding when DNA synthesis is disturbed. For this purpose we compared the level of ssDNA when several checkpoint proteins are knocked down during normal and perturbed DNA synthesis. Level of ssDNA was evaluate using live cell imaging of human cell lines that express EGFP tagged subunits of RPA protein under their native regulatory sequences and near physiological expression level. We show that EGFP-RPA foci are not visible during normal DNA replication, that indicate that the synchronisation of DNA unwinding and synthesis does not allow generation of vast stretches of ssDNA. In contrast, extensive RPA foci were detected when DNA synthesis was inhibited by aphidicolin or hydroxyurea, suggesting that the inhibition of DNA synthesis does not lead to simultaneous inhibition of replicative unwinding. However, the defined size of the RPA70-EGFP foci suggests that cells possess a mechanism that prevents massive and uncontrolled DNA unwinding when synthesis is disturbed.

We next checked whether the S-phase checkpoint prevents massive DNA unwinding. Our results show that inhibition of S phase checkpoint by caffeine or by knocking down the ATR checkpoint kinase leaded to massive increase of ssDNA, indicated by a burst in the size of RPA70-EGFP throughout the entire nucleus when DNA synthesis is prevented by aphidicolin. However, the knock-down of ATR without inhibition of DNA synthesis by aphidicolin did not lead to RPA70-EGFP foci formation. Our results suggest that ATR checkpoint kinase participates in a mechanism that prevents massive DNA unwinding when DNA synthesis is inhibited. In addition, we found that three S-phase checkpoint mediator proteins are also involved in regulation of DNA synthesis and unwinding. The RNA interference of Tipin, Claspin and Tim1 leaded to massive increase of ssDNA, when DNA synthesis was inhibited by aphidicolin. Such phenotype was not observed when control esiRNAs were used. The RNA interference of the three proteins without inhibition of DNA synthesis did not lead to RPA70-EGFP foci formation. Our results suggest that Tim, Tipin and Claspin also participate in a mechanism that prevents massive DNA unwinding when DNA synthesis is inhibited.

Further experiments will be required to reveal how the ATR kinase and its mediators Tim, Tipin and Claspin contribute to the inhibition of replicative unwinding when DNA synthesis is inhibited. Nevertheless, a possible model, combining our results and the available data, can be used to explain the regulation of DNA unwinding. When cells are treated with HU, aphidicolin or DNA is damaged, DNA synthesis slows down and ssDNA regions are generated. RPA attaches to these ssDNA regions and promotes the ATR-ATRIP binding. The interaction of Claspin/Tim1/Tipin with ATR and/or their subsequent phosphorylation by ATR kinase could interfere with the ability of Claspin/Tim1/Tipin to bind MCM complex and thus to inhibit its helicase activity.

CHILE

Title: Role of the cyclooxygenase-2 (COX-2) and caveolin-1 in CK2-induced metastasis
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Abstract: β-Catenin (β-cat) is crucial in canonical Wnt pathway. Its activation implicates stabilisation and nuclear import of β-cat, following increased expression of survivin and cyclooxygenase-2 (COX-2), which promote viability. Constitutive activation is linked to development and progression of many cancers. CK2 stabilises β-cat and precludes its degradation, promoting survivin expression, viability and apoptosis resistance. Also, caveolin-1 (cav-1) is a tumour suppressor in many cancers. We already showed that cav-1 down-regulates survivin and COX-2 expression by inhibiting β-cat-dependent transcription, but this requires E-cadherin (E-cad). E-cad is a tumour suppressor by retaining β-cat at cell surface and precluding gene expression. Cav-1 also associates with β-cat and E-cad at surface forming the CBE complex. We proposed that CK2 promotes invasion by augmenting COX-2 expression, prostaglandin E2 (PGE2) production and disruption of CBE, thereby enhancing β-cat activity. Our aim was to identify the mechanism by which CK2 disrupts CBE complex and how this enhances invasion. We found that CK2 promoted expression of COX-2 and production of PGE2. Also, PGE2 disassociated cav-1 from CBE complex, which induced β-catenin activity. Thus, insights generated from this study allow postulate the CK2/COX-2/PGE2 axis as a novel promising therapeutic target for anticancer therapy.
Objectives:
Our general objective was to define the mechanism of CK2-mediated disruption of caveolin-1/ β-catenin/E-cadherin (CBE) complex formation and how this enhances invasion. Specific objectives were as follows:
(i) Determine the effect of CK2 on COX-2 expression/PGE2 production and disruption of CBE complex;
(ii) Investigate the role of CK2/COX-2/PGE2 axis in cancer progression;
(iii) Determine whether PGE2-enhanced viability is modulated by the presence of caveolin-1.
Results Obtained:
Protein kinase CK2 promotes cancer cell viability via up-regulation of cyclooxygenase-2 expression and enhanced prostaglandin E2 production. Our results demonstrate that CK2 enhances cell viability and link these events to up-regulation of COX-2 expression via the Wnt/β-catenin pathway in human colon and breast cancer, as well as embryonic cells. Decreases in CK2 activity, achieved either by pharmacological inhibition or expression of a dominant negative variant, significantly reduced cell viability and this effect was linked to reduced expression of COX-2. Additionally, PGE2 supplementation prevented decreases in cell viability following CK2 inhibition. Therefore, these data provide important new insights to the role of CK2 as an oncogene by identifying COX-2/PGE2 as key components downstream of this kinase. Given the relevance of CK2 activity for tumour cell viability, these findings raise the interesting possibility of improving available cancer therapies by selectively inhibiting CK2.
Effects of PGE2 in viability and invasiveness are mediated through a multiprotein CBE complex present in colon cancer cells. We demonstrated that a multiprotein complex formed by caveolin-1, β-catenin and E-cadherin (named CBE) is present at the cell surface of several colon cancer cells. PGE2 binding to its cognate receptor EP2 is able to induce the disassociation of caveolin-1 from the CBE complex, which apparently does not affect the association of β-catenin to E-cadherin. However, caveolin-1 disassociation promotes the release of β-catenin from its interaction with E-cadherin, thereby increasing stability and nuclear activity of β-catenin on cancer-related genes, like survivin (and COX-2 itself), which ultimately enhances survival and tumour progression of colon cancer cells. Importantly, similar results were observed with an EP2 agonist, butaprost, confirming this way the participation of the EP2 receptor in this process.
Results Unforeseen in the Original Project:
The participation of the cognate PGE2's receptor EP2 in the mechanism of activation of β-catenin activity by protein kinase CK2 was the most unforeseen result in this project. When a specific EP2 agonist was used, butaprost, it was sufficient to produce essentially the same effects observed with PGE2 in colon cancer cells: disassociation of caveolin-1 from CBE, release of β-catenin from its binding to E-cadherin, and increased expression of survivin irrespective of overexpression of caveolin-1.
Publications:
Yefi, R., Ponce, D.P., Niechi, I., Silva, E., Cabello, P., Rodriguez, D.A., Marcelain, K., Armisen, R., Quest, A.F.G., Tapia, J.C. Protein kinase CK2 promotes cancer cell viability via up-

regulation of cyclooxygenase-2 expression and enhanced prostaglandin E2 production. 2011. J. Cell. Biochem. **112**, 3167–3175

CUBA

Title: Knowledge generation using a biological data warehouse system
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Abstract: Modern biology, medicine and biotechnology use many highly productive data collection technologies that often provide several thousands of measured data in a single experiment. The bottleneck is the interpretation of the data, which requires the simultaneous use of a many different, often noisy and redundant databases. Data integration is a one of the main requirement to develop System Biology applications. In our group we have integrated different data sources as part of our application BisoGenet with the purpose of building biological networks (see Martin et al., BMC Bioinformatics 2010, 11:91). The present project aims to further develop data integration oriented to the studies of genes and its relation to disease development. As a main project results will be generated a web application allowing complex queries related to genes functional relations and its implication in disease development.

Objectives:

The proposed research project had the following two main objectives: (i) further development of a warehouse system to integrate data on gene-diseases relationships and microRNAs and theirs target genes (ii) building web tools to use the warehouse system developed as a framework to support bioinformatics research and development.

Results Obtained:

A search engine (web application) for SysBiomics data warehouse (http://bio.cigb.edu.cu/SBweb/) was developed. SysBiomics database compiles and integrates data from multiple public sources that include: general notes about genes and proteins (NCBI, UniProt), functional notes (KEGG, GO), interactions between genes/proteins (DIP, BIND, HPRD, BioGrid, MINT, Intact) and gene-disease association information (OMIM, GAD, CTD). The system can be used to infer new biological relations applied to real biological projects depending of course on the experimental design.

IRAN

Title: DNA methylation pattern changes and detection of breast cancer

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Abstract: Serum DNA methylation pattern analysis may offer a non-invasive method to diagnose cancer in early stages with low cost. It also has the potential to become a tool for analysing the effects that environmental factors have on developing cancer cells. In this study, we aimed to compare methylation pattern of serum DNA from blood, cancerous and normal tissues of patients from the Babol province of Iran, as the incidence of breast cancer among women living in this region is significantly higher than national average. Subsequently, to determine if the methylation pattern of serum DNA reflects the progression of cancer in these patients, and, later on to see if these patterns have been affected by environmental factors specific to the agricultural regions of Iran. In this report we present the preliminary results gathered through this one-year grant.

Objectives: Our main objective was to determine the methylation pattern in 6 selected genes in serum circulating DNA known to be involved in breast cancer development.

Results Obtained:

Specimens used in the phase I of the study were obtained from tumour, matched normal and serum samples of 18 patients collected form Babol hospitals (from a northern region of Iran) and also six samples (selected patients from a northern region of Iran) purchased from Iran Tumour Bank. DNAs were extracted from breast tumour/normal tissue using Bioneer and Sinaclon kits. Serum DNA was extracted using Qiagen kit. For bisulfite treatment, DNA samples were treated according to EpiTect Bisulfite kit. Quality and quantity of the DNAs and converted DNAs were measured by nanodrop spectrophotometer. Primers for *NANOG* and *CTSL 2* genes were designed in CpG islands using online and offline software (Methprimer, NCBI and Oligo). Primer sequences for five other genes (RASSF1A, 14-3-3, Caspase8, WIF1, SOCS3) were obtained from published research articles. The specificity of each primer for methylated and unmethylated CpG regions were tested using EpiTect PCR Control DNA set. MSPCR tests were performed for *NANOG*, *RASSF1A*, *Caspase8*, *14-3-3* and *CTSL 2* genes using HotStar Taq Plus Master Mix kit. Since none of the primers used for SOCS3 lead to DNA amplification, we chose instead WIF1 gene, which is known to be differentially methylated in beast cancer (BMC Cancer 2009, 9:217, doi: 10.1186/1471-2407-9-217).

We tabulated the data and analysed them using Fisher test to identify potential significant differences between the tumour and normal samples. The results demonstrate that the methylation status for all genes except WIF1 is not significantly different between tumour and normal tissues. Specifically, for WIF1 gene no methylation was observed in the tumour tissue, while 28% of normal tissues were methylated. This is quite a significant finding and potentially can be used as a marker. However, more samples have to be analysed to confirm this finding.

Results Unforeseen in the Original Project:

A surprising finding in the study was the observation of PCR amplification of methylated and unmethylated DNA with specific primers in both tumour and normal tissues. Although in the original proposal we were planning to analyse the methylation patterns by sequencing specific areas for these 6 genes in breast cancer tumour, we were unable to do this due to lack of funding and difficulties which arose as a result of economic embargo. Therefore, we were forced to modify our strategy and analyse the single nucleotide changes in the genes and use MSPCR technique, which is a very limited technique in analysing the methylation status and not patterns (as we originally planned). In the final analysis we can only hypothesise about this mixed results. The nonspecific amplifications may be due to (i) contamination of tumour and/or normal tissues, (ii) heterozygosity of genes studied, or (iii) lack of specific binding of primers for reasons unknown to us.

Although another objective of this phase of the study was to determine the methylation pattern in 6 selected genes in serum circulating DNA involved in breast cancer development, we were unable to isolate enough DNA for each serum sample to perform MSPCR and bisulfite treatment. This may have been due to (i) technical difficulties, (ii) non-standard preservation method of serum at the site of the sample collection centre or (iii) rapid degradation of DNA in serum. Therefore, in this phase of the study we have reported the methylation results from tissue samples (tumour and normal).

MEXICO

Title: Paralogues evolution: relocalisation and expression profiles
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Abstract: *Saccharomyces cerevisiae* genome arose from a complete duplication of eight ancestral chromosomes (Whole Genome Duplication WGD); normal ploidy was recovered due to the loss of 90% of duplicated genes, and the retention of 8% duplicates. Gene duplication, retention and the subsequent divergence of paralogous pairs play an important role in the evolution of novel gene functions. Retained duplicates can provide an increased dosage of the same product or go through a process of neo or sub-functionalisation. The lineage which gave rise to *Kluyveromyces lactis* (*K. lactis*) and *Lachancea kluyveri* (*L. kluyveri*) diverged before the WGD event; therefore, *K. lactis* and *L. kluyveri* genomes do not harbour the duplication blocks present in *S. cerevisiae*, and can be regarded as "ancestral type" yeasts. In this study, we have analysed the role of differential gene expression, subcellular relocalisation and formation of hetero-oligomeric isozymes in the functional diversification of paralogous isozymes as compared with the orthologous "ancestral type" genes or enzymes. Our results show that paralogous pairs display opposed regulatory profiles determined by transcriptional regulators which can alternatively act as positive or negative modulators, that subcellular localisation modulates paralogous function and that hetero-oligomeric enzymes with peculiar kinetic properties are formed *in vivo*.
Objectives:
Determine the impact of expression divergence (*cis-trans* interaction), chromatin organisation and subcellular relocalisation on the acquisition of distinct functions of a selected group of *S. cerevisiae* paralogues genes as compared with the functional characterisation, expression profile and subcellular localisation of the *L. kluyveri* and *K. lactis* single copy orthologous genes.
Results Obtained:
Considering previous work from our group, an *in silico* subcellular localisation analysis was carried out, and a selected collection of paralogous genes belonging to the duplicated blocks generated after the WGD event, was chosen for this study: *ALT1-ALT2, BAT1-BAT2, LEU4-LEU9* and *GDH1-GDH3*.
ALT1-ALT2: Our results show that although *ALT1* and *ALT2* encode 65% identical proteins, only Alt1 displays alanine aminotransferase activity; in contrast, *ALT2* encodes a catalytically inert protein. *ALT1* expression is alanine-induced, showing a regulatory profile of a gene encoding an enzyme involved in amino acid catabolism, in agreement with the fact that Alt1 is the sole pathway for alanine catabolism present in *S. cerevisiae*, an *alt1Δ* mutant does not grow on alanine as sole nitrogen source. Conversely, *ALT2* expression is alanine-repressed, suggesting a biosynthetic role; surprisingly, *ALT2* encoded-protein has no alanine aminotransferase enzymatic activity, and an *alt2Δ* mutant shows wild type phenotype. A double *alt1Δ alt2Δ* mutant is not an alanine auxotroph indicating the presence of an alternative biosynthetic pathway (s). In the ancestral-like yeasts *L. kluyveri* and *K. lactis*, alanine aminotransferase activity was detected, alanine biosynthesis and catabolism are only partially impaired in *Lkalt1Δ* and *Klalt1Δ*, mutants, suggesting the existence of alternative alanine metabolic pathways. *S. cerevisiae alt1Δ* mutants were complemented by *ALT1*, *KlALT1* and *LkALT1* and not by *ALT2*. Transcriptional regulation analysis showed that Gcn4 activates expression of both, *ALT1* and *ALT2*, that *ALT1* expression is negatively determined by Rtg3, Nrg1and Hda1, while that of *ALT2* is negatively determined by Rtg3, Nrg1, Tup1 and Hda1. Regulation by Hda1 indicates a role of chromatin organisation, which is currently being analysed. The fact that Rtg3 acts as a negative regulator is novel since this transcriptional modulator has been regarded as activator. The fact that the sole lack of Rtg3 or Nrg1 results in derepression suggests these proteins are forming a complex. We are currently working on the analysis of co-inmunoprecipitates with differentially tagged strains to determine whether the predicted Rtg3-Nrg1 complex exists.
ALT2 retention poses the questions of whether the encoded protein plays a particular function, and if this function was present in the ancestral gene. It could be hypothesised that *ALT2* diverged after duplication, through neofunctionalisation or that *ALT2* function was present in the ancestral gene, with a yet undiscovered function. To analyse this matter and to compare kinetic properties of the isozymes and determine their oligomeric organisation, Alt1, *Kl*Alt1 and *Lk*Alt1 were purified as HIS-tagged recombinant proteins from *E. coli*, kinetic analysis is under way. So far, we have not been able to purify Alt2, the induced protein was precipitated forming inclusion bodies, thus its oligomeric organisation was not assessed.

In order to address the question of the lack of aminotransferase function of Alt2, we are now constructing Alt1-Alt2 chimeric proteins to identify the region(s) involved in Alt1 activity, which are lacking or modified in Alt2. Site directed mutagenesis of residues predicted to be involved in oligomeric interactions will also be obtained.

BAT1-BAT2: *K. lactis Kl*Bat1 is a bifunctional aminotransferase, which participates in the biosynthesis and catabolism of branched chain amino acids (BCAAs). This dual role has been partially distributed in *S. cerevisiae* Bat1 and Bat2 paralogous proteins, probably due to diversification of expression patterns: *BAT1* is highly expressed under biosynthetic conditions, while *BAT2* expression is highest under catabolic conditions. Since Bat1 is mitochondrial, while Bat1 is cytosolic, it had been proposed that differential localisation could affect their function. In this study, as result of the collaboration, supported by this project, with Dr. Jure Piskur from Lund University (Sweden), Mirelle Flores made two stages during which re-localised mutants were constructed, and physiological analysis of wild type and mutant strains under aerobic and anaerobic conditions was carried out. Our results show that under aerobic conditions Bat1 and Bat2 respectively play a preferentially biosynthetic and catabolic role, which does not depend on the subcellular localisation, while under anaerobic conditions, when Bat2 is localised in the mitochondria, it acquires a biosynthetic character partially substituting lack of Bat1. Results indicate that when Bat1 and Bat2 are co-localised in the mitochondria in glucose + valine-ileucine-leucine (VIL) under aerobic conditions a negative interaction could be established suggesting that differential localisation could have been selected to avoid Bat1-Bat2 interactions.

During this study, we established a collaboration with Dr. Joseph Strauss from BOKU University in Vienna (Austria) and James González made a stage there during which he carried out a detailed analysis of the transcriptional regulation of *BAT1* and *BAT2*. It was found that *BAT1* expression is not determined by the quality of the nitrogen source and is repressed by the combined action of valine-isoleucine and leucine (VIL). This repression could be triggered by the negative effect of Put3 and through nucleosome sliding. *BAT1* transcriptional activation is determined through the action of Leu3 and Gcn4. *BAT2* expression is repressed in glutamine and induced in secondary non-repressive nitrogen sources such as GABA and VIL. *BAT2* transcriptional activation is achieved through the combinatory action of the modulators Leu3, Gln3 and Ure2, while repression in glutamine is achieved through a novel Gln3-independent mechanism determining response to the nature of the nitrogen source, triggered through the negative action of Leu3. Nucleosome displacement by the Swi/Snf chromatin-remodeling complex could also play a role in the Leu3-dependent response to the nutrient signal of the quality of the nitrogen source.

Results Unforeseen in the Original Project:

LEU4-LEU9: To analyse whether oligomeric paralogous mitochondrial enzymes can constitute heterooligomeric isoforms *in vivo*, the *LEU4-LEU9* paralogous pair encoding α-isopropylmalate synthase dimeric enzyme involved in leucine biosynthesis was selected. Bimolecular Fluorescence Complementation (BIFC) analysis showed Leu4-Leu9 interaction by reconstituting fluorescence from Leu4 or Leu9 proteins tagged with yECitrine amino or carboxy half-epitopes. *LEU4* and *LEU9* were independently or simultaneously expressed from the *ENO2* promoter and Leu4-Leu4, Leu9-Leu9 and Leu4-Leu9 enzymes were purified from isolated mitochondria. Results indicate that Leu4-Leu9 hetero-oligomerisation is preferred to homo-oligomerisation, that the three isozymes show differential leucine inhibition, showing that paralogous proteins can form hetero-oligomeric isoforms *in vivo* with peculiar kinetic properties. Orthologous "ancestral type" genes from *K. lactis* were identified, and it was found that *LEU4* is duplicated (*KlLEU4* and *KlLEU4BIS*). Both genes were cloned and single mutants were obtained. These mutants show wild type phenotype indicating a redundant function. *KlLEU4BIS* shows syntheny with *LEU4* and *LEU9* and is able to complement the braditrophic phenotype displayed by *leu4Δ* mutants.

GDH1-GDH3: Supported by this grant, Edson Robles made a stage in Dr. Jure Piskur's laboratory in Lund University, during which he established a novel protocol to *in vitro* analyse the formation of hetero-oligomeric isoforms of the hexameric Gdh1 or Gdh3 glutamate dehydrogenase enzymes. *In vitro* formation of hetero-oligomers was carried out by reconstituting mixtures of Gdh1, Gdh3 and *Lk*Gdh1 monomers. The hybrid proteins obtained were kinetically characterised and identification was carried out by mass spectroscopy MALDI-TOFF, using 13C, 15N-labeled peptides as internal standards. The Gdh1, Gdh3, *Lk*Gdh1 recombinant proteins showed classical Michaelis Menten kinetics when the substrate concentration was varied. Recombinant proteins were denatured together in guanidinium chloride and then mixed in a 1:1 ratio (Gdh1/Gdh3, Gdh1/ *Lk*Gdh1 and, Gdh3/ *Lk*Gdh1). The renatured mixture was separated in an affinity column. This study shows evidence indicating the presence of functional heterooligomers formed between paralogues and ortologues proteins. The formation of hybrid proteins from paralogous proteins results in the formation of a collection of isozymes with distinct and unique affinity for Ni columns, more stable and with a higher capacity to utilise NADPH than hybrid proteins from orthologues proteins.

Concluding remarks: Gene duplications and subsequent divergence play an important role in the evolution of novel gene functions. This study has focused on the analysis of elements involved in the process of sub or neo-subfunctionalisation. Results presented in this study uncover new elements triggering functional diversification: (i) Formation of hetero-oligomeric proteins influences

the number of isozymes that can be built up with peculiar kinetic properties, conferring versatility to the physiological role played by the duplicated genes; (ii) Expression divergence analysis revealed that a given modulator can play a positive or negative role which is independent of small molecules acting as co-inducers or corepressors, suggesting the existence of an intrinsic capacity of a modulator to recruit repressing or activating machinery; (iii) Subcellular relocalisation of paralogous proteins as compared to that of the ancestral type orthologous protein affects functional diversification. It is worth mentioning that new questions such as: How does the preferential formation of hetero-oligomers vs homo-oligomers is achieved? What determines whether a regulator can act as positive or negative effector? have arisen from our study highlighting the importance of the financial support received from ICGEB.

Publications:

Peñalosa-Ruiz, G., Aranda, C., Ongay-Larios, L., Colon, M., Quezada, H., Gonzalez, A. Paralogous *ALT1* and *ALT2* retention and diversification have generated catalytically active and inactive aminotransferases in *Saccharomyces cerevisiae*. 2012. PLoS One **7(9)**, e45702

López, G., Quezada, H., Duhne, M., González, J., Lezama, M., El-Hafidi, M., Colón, M., Martínez de la Escalera, X., Flores-Villegas, M.C., Scazzocchio, C., DeLuna, A., González, A. Diversification of paralogous α-isopropylmalate synthases by modulation of feedback control and hetero-oligomerization in *Saccharomyces cerevisiae*. 2015. Eukaryot. Cell (Epub ahead of print)

PAKISTAN

Title: Structural and functional studies of C-terminal hydrophilic tail of the human zinc transporter Znt8

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Abstract: Zinc is an essential metal cation in cellular processes, since it acts as co-factor for many enzymes and is part of transcription factors and other proteins. Zinc transport across biological membrane, is mediated by specific membrane proteins. One of these, human zinc transport protein hZnt8, is expressed in pancreas beta cells where it provides zinc for processing of insulin. In 2007 a mutation (R325W) in hZnt8 has been linked with pathogenesis of Type 2 diabetes and determination of autoantibody epitope specificity in Type 1 diabetes. Bioinformatic analysis suggests that Arg325 is located in cytolplasmic C-terminal tail of hZnT8. Thus, we studied the structure and function of wild type cytolplasmic C-terminal domain of hZnt8 and of that containing R325W mutation. To this end, we have successfully over-expressed hZnt8 and hZnT8 (R32W) C-terminal cytoplasmic domain in *Escherichia coli* in fusion with oligohistidine tag and maltose binging protein. Native hZnT8 and hZnT8 (R325W) have been purified to homogeneity in tetrameric state by combination of Nickel and amylose affinity, ion exchange and size exclusion chromatography. hZnT8 (R325W) domain binds to Zn^{2+} ions with similar affinity as that of hZnT8. Moreover, spectroscopic studies revealed that R325W mutation did not produce any significant change in global structure of the domain. Overall, this study suggests that R52W mutation in hZnT8 may be tolerated in diabetic patient.

Objectives:

The main objective of this research project is to study the structure and function of the so-called C-terminal cytoplasmic domain of hZnt8 with perspective of finding a link between R325W mutant of hZnt8 and diabetes. This was achieved through a number of objectives:

(i) Soluble expression of C-terminal cytoplasmic domain of hZnt8 (wild type and mutant R325W) in *E. coli* through various strategies;

(ii) Purification of monodispersed and homogenous hZnt8 and hZnT8 (R325W) domain;

(iii) Analysis of zinc binding to hZnt8 and hZnT8 (R325W) domain;

(iv) Structural analysis of hZnt8 and hZnT8 (R325W) domain

Results Obtained:

Over-expression of wild type hZnT8 and variant hZnT8 (R325W) C-terminal domain in fusion with N-terminal His_8-MBP-TEV soluble partner was obtained in *E. coli* Rosetta 2 (DE3), using constructs pMR19MR and pMR19MR-R325W, respectively. Over expressed domains were purified by Ni-NTA affinity chromatography. Native hZnT8 and hZnT8 (R325W) domain were obtained by treating purified His_8-MBP-TEV-hZnT8 and His_8-MBP-TEV-hZnT8 (R325W) proteins with lab-made TEV protease followed by removal of oligohistidine tagged proteins by combination of ion-exchange, reverse nickel and maltose affinity methods. The molecular identity of the purified hZnT8 domain was confirmed by mass spectroscopy that gave a molecular mass of 9.828 kDa. We expect that variant hZnT8 (R325W) is also intact as its size is equivalent to wild type hZnT8, as analysed by SDS-PAGE. Size exclusion chromatography revealed that hZnT8 and hZnT8 (R325W) domain exist in homogenous tetrameric form in solution, as indicated by an apparent molecular mass of 40 kDa. Further, electron microscopy analysis of both wild type and variant domain also suggest that purified proteins are free of any high molecular mass aggregates. To get such a high protein purification quality is one of the prerequisite for structural and functional characterisation of any target.

In order to examine that tetramer state of the hZnT8 domain may not be due to disulfide linkage between -SH of three cysteine residues present in the domain, we have performed size exclusion chromatography under reducing conditions using 3 mM beta-mercaptoethanol in 20 mM Tris-Cl pH 7.5 containing 150 mM NaCl. Similar SE-chromatogram was obtained as was under native condition showing that tetramer state of the domain is due to specific interdomain interactions and is not because of any disulfide linkage. It has been believed that C-terminal cytoplasmic domain of zinc transport proteins exists in oligomeric state by coordinating with Zn^{2+} ions. To verify this, SE-chromatography analysis was conducted in the presence of 5 mM EDTA, EDTA chelates Zn^{2+} ions, which result in dissociation of tetramer complex into monomer domain.

To assess Zn^{2+} binding affinity to hZnT8 and hZnT8 (R325W) domain, we exploit the intrinsic fluorescence of proteins. Addition of saturating concentration of Zn^{2+} to hZnT8 and hZnT8 (R325W) samples induced a significant quenching of ~9%. The quenching increased with increasing Zn^{2+}

concentration in an approximately hyperbolic manner. The data was fitted to one site binding model that produce dissociation constant (K_d) 0.04±0.006 μM and 0.08±0.02 μM for Zn^{2+} binding to hZnT8 and hZnT8 (R325W) domain, respectively, revealing that wild type and mutant domain binds to Zn^{2+} with significant affinity and R325W mutation may be tolerated in type 2 diabetes patient.

To investigate whether hZnT8 and hZnT8 (R325W) display similar structural features, CD analysis was performed. The CD spectrum of hZnT8 was found almost identical to that obtained for hZnT8 (R325W), particularly in the region between 200–240 nm that reflects the secondary structure propensities in proteins. Thus, it is likely that R325W mutation did not lead to any significant structural changes in the domain. Moreover, addition of 1 mM Zn^{2+} to hZnT8 or hZnT8(R325W) did not produce any prominent structural change indicating that zinc binding is independent of any alteration in global structure of the wild type and mutant domain. Finally to check the thermally stability and refolding ability, CD spectra were recorded for hZnT8 and hZnT8 (R325W) domain that were heated up to 90 °C followed by an attempt of refolding by cooling to 25 °C, show that domains could not be refolded after thermal unfolding.

There are no X-ray or NMR 3-dimensional structures available for full hZnT8 or hZnT8 C-terminal cytolplasmic domain. Thus, to estimate secondary structure of hZnT8 and hZnT8 (R325W) domain, we have used data from FT-IR spectroscopy, which indicate that both domains fold into mixed secondary structure components. To provide quantitative estimate of the alpha-helical and beta-sheet content of hZnT8 and hZnT8 (R325W) domain, the measured amide I region of respective spectra could be reproduced by a fit of seven and nine components respectively, dominated by those indicative of alpha-helix and beta-sheet. Quantitative analysis of the spectra predicted the presence of ~ 29-27% alpha-helix and ~ 26-24% beta-sheet structures for both wild and mutant domain. These results also suggest that R325W mutant did not lead to any major structural change in C-terminal cytolplasmic domain of hZnT8.

Crystallisation of native wild type and variant (R325W) hZnt8 domain is being pursued. Crystallisation trials were set up using a range of crystallisation screens such as Crystal Screen I, Crystal Screen II, JCSG Screen, Structure Screen, PACT Suite, PACT premier, Xtal Screen 1+2, ComPAS Suite, Natrix Screen, Index Screen, Classic Screen, Classic II screen, pH Clear Screen and Morpheus screen at Elettra Synchrotron facility, during the visit of a Ph.D. student to Trieste, as well as at our home Institute, and are being examined.

Results Unforeseen in the Original Project:

Cytoplasmic C-terminal domain of bacterial homologue of hZnT8 folds as tetramer, however we have found that hZnT8 domain folds as tetramer, which is an unexpected and surprising finding.

Publications:

Ullah, R., Shah, M.A., Tufail, S., Ismat, F., Imran, M., Iqbal, M., Mirza, O., Rhaman, M. Activity of the human rhinovirus 3C protease studied in various buffers, additives and detergents solutions for recombinant protein production. 2016. PLoS One, **11(4)**, e0153436

Ullah, R., Shehzad, A., Shah, M.A., De March, M., Ismat, F., Iqbal, M., Onesti, S., Rahman, M., McPherson, M.J. C-terminal domain of the human zinc transporter hZnT8 is structurally indistinguishable from its disease risk variant (R325W). 2020. Int. J. Mol. Sci. **21**, 926

PERU

Title: Transplanted proteins as new vaccine carriers

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Abstract: Traditional recombinant vaccines are usually composed of rapid degradable and processed proteins, while epitope/peptide vaccines are composed of linear arrangements of immunogenic and protective peptides linked to carriers. The efficacy of an antigen as a vaccine depends partly on the duration of its exposure to the immune system (directly proportional to its life span in circulation) as well as the immune system's capacity to recognise the three-dimensional immunogenic epitopes.

Current approaches to design peptide vaccines basically consist on the construction of oligopeptides comprising linear arrangements of immunogenic and protective epitopes. These oligopeptides can be obtained via recombinant bacteria expression systems and are usually delivered conjugated to carriers. These molecules do not usually have a long life span in the circulatory system, and therefore several doses are required in order to elicit immunity.

No special attention has ever been given to the 3D structure of the immunogenic epitopes within the conjugated carrier. We propose a novel strategy to deliver immunogenic epitopes that uses stable serum proteins on which selected immunogenic epitopes are "transplanted" into the scaffold without perturbing the original 3D structure or stability. Careful design of this transformed serum proteins will provide to the selected transplanted epitopes a physical-chemical environment that favours a secondary structure and an orientation to the solvent similar to those of the native protein from which the epitope was obtained. The preservation of the global structure of a transplanted serum protein, would permit the conservation of its long life span in plasma, which will end in a gradually and slow presentation of the epitopes to the immune system, mimicking a continuous micro- immunisation (not achieved by current epitope vaccines). We hypothesise this is advantageous to elicit a stronger immunity.

This proposal seeks to demonstrate the "proof of principle" by which an immunogenic epitope transplanted on the scaffold of a serum protein is able to elicit a stronger immune response than if it is only conjugated. We plan to use the well-researched mice-influenza A hemagglutinin model to verify our hypothesis. In the last decade, several hemagglutinin epitopes have been identified as protective against influenza-A virus. Our group has confirmed in a theoretical computer model that the mouse albumin is the best receptor with a structure suitable for this transplant "molecular surgery". We plan to transplant three influenza-A hemagglutinin epitopes into the mouse albumin, measure its capacity to elicit neutralising antibodies, and compare with the effect of the epitopes traditionally conjugated to albumin. If succeeding in demonstrating the "proof of principle", we believe that this general approach of using stable serum proteins as targets to transplant immunogenic epitopes could open a new generation of vaccines, to be used in multiple infectious diseases.

Objectives:

We hypothesise that a serum protein converted to carry immunogenic epitopes transplanted on its surface, can induce a better antibody response and better protection than epitopes delivered by chemically bound (cross-linked) to carrier proteins or epitopes alone without any chemical alteration.

The major goal of this project is to test an alternate approach to design epitope-vaccines, by delivering immunogenic/protective epitopes transplanted onto the surface of native serum proteins instead of conjugated to carriers. These transplanted serum proteins will be able to expose one or more immunogenic/protective epitopes, preserving their original 3-dimensional structure and orientation to the solvent without altering the structure and solubility of the serum protein. The preservation of the 3D structure will ensure a long lifespan in circulation, which would permit a gradually and slow presentation of the epitopes to the immune system, mimicking a continuous micro-immunisation. We will identify domains located at the surface of the serum protein that are similar to the epitope structure. Then we will replace these domains with the selected epitopes, thus constituting a transplanted protein.

To demonstrate the "proof of principle" of this hypothesis, we will create a transplanted mouse albumin, with selected immunogenic epitopes of the influenza A hemagglutinin. We will compare the immunogenicity in a mice model immunised with the transplanted albumin and compared with an immunisation with epitopes cross-linked to mouse albumin.

Results Obtained:

We were able to produce soluble and functional mouse albumin, both wild type and transplanted with a selected epitope from influenza A hemagglutinin. Initially and using structural bioinformatics and *ab initio* computing approaches, we determined the best position of the mouse albumin surface to receive the transplanted epitope. We tested both the feasibility of folding and the thermodynamical stability. The coding gene of mouse albumin was synthetically produced with the codon usage optimised for *E. coli*. The recombinant transplanted albumin included a His-tag in the carboxy terminal to achieve purification with a His-trap affinity chromatography.
We also produced recombinant wild type mouse albumin with a His-tag in the carboxy terminal. After producing the cDNA of the mouse albumin, we had to change a codon with site directed mutagenesis to eliminate an undesirable restriction site. Similarly, the recombinant wild type mouse albumin was expressed in *E. coli* and purified with His-tag affinity chromatography. The recombinant wild-type mouse albumin was used to cross-link the hemagglutinin epitope on its surface.
Finally, we immunised six groups of ten mice, each with one of the following antigens: (1) albumin-(His tagged) transplanted with epitope, (2) wild type albumin-(His tagged) crosslinked with epitope, (3) wild type albumin-(His tagged) mixed with epitope; and as controls, (5) wild type albumin-(His tagged) and (6) commercial purified wild type mouse albumin (i.e., with no His-tag).
In all cases, the commercial, wild type, and epitope-transplanted recombinant albumins showed to be soluble and functional. Using atomic absorbance we confirmed that each recombinant protein was able to bind Zn in the expected ratio protein:metal.
The three most important results obtained were:
(i) The albumin transplanted with the epitope was correctly folded, functional and stable;
(ii) Antibodies were raised against the His-tag of the recombinant albumins in all the cases;
(iii) Antibodies against the influenza hemagglutinin epitope were elicited. This reaction was observed after adjusting for the reaction against the His-tag end. The higher titers were observed after the second and third dose of the vaccination. The transplanted epitope showed the higher titers followed by the crosslinked epitope and in least extend with an undetected level, the epitope mixed with the recombinant albumin.

Results Unforeseen in the Original Project:

An unexpected result was observed. We found that sera from animals vaccinated with recombinant albumin His-tag protein induced antibodies that reacted against the commercial wild type purified albumin (i.e., non His-tagged). However, animals vaccinated with commercial wild type purified albumin did not produce antibodies against this antigen. Therefore a level of auto-immunity was generated. It is important to remark that this level of autoimmunity was neither lethal nor toxic. All the animals survived for at least 8 months after the first inoculation. A possible explanation for this situation is that antibodies raised against the His-tag are also recognising partially the carboxy-terminal region of the mouse albumin. Therefore, these antibodies are able to react and bind to the purified wild type albumin.
Main conclusion: This study demonstrates the proof of concept that an epitope transplanted into the surface scaffold of a serum protein, and thus preserving its native structure, and degrading gradually while being presented to the immune system, induces higher levels of antibodies compared to epitopes cross-linked randomly on the surface of the same serum carrier.
Future plans: We still have the animals alive, and in the next weeks we will apply a last dose of vaccine. We plan to measure immune memory. We hypothesise that animals vaccinated with the transplanted albumin may show a stronger memory compared to the other antigens.
Before the sacrifice of the animals, we plan to purify the PBMC cells in order to test for a cellular response against the single epitope used in the transplantation and in the crosslinking. We will run an ELISPOT for this.
It is important to re-evaluate a new antigen, without the His-tag end. For this we plan to produce a recombinant transplanted albumin with the His-tag added to the amino-terminal region. After that we will cut the His-tag and further purify with a size exclusion chromatography followed by concentration with ultra-filtration.

RUSSIA

Title: The role of transcription factor SAYP in nuclear receptor dependent transcription
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ICGEB Contract No.: CRP/10/015
ICGEB Reference No.: CRP/RUS10-02
Abstract: This project is aimed to consider the role of metazoan coactivator SAYP in nuclear receptor-driven gene activation in the ecdysone cascade of Drosophila. On the first stage of the project we demonstrated the interaction of SAYP with DHR3 nuclear receptor of ecdysone cascade and characterised their interaction. During the period of the grant we demonstrated that DHR3 together with SAYP is essential for transcription of SAYP-dependent genes. They both participate in recruitment of the BTFly (Brahma and TFIID) supercomplex to promoter of the gene. Moreover SAYP together with Brahma complex are essential for organisation of mRNA pausing at promoters of transcriptionally repressed genes and organise the chromatin barrier ahead of the paused mRNA. During active transcription, SAYP is essential for Ser2 phosphorylation and transcription elongation.

Objectives:

The project was aimed:
(i) to study the role of SAYP with DHR3 nuclear receptor of ecdysone cascade and characterised their interaction;
(ii) to investigate the role of SAYP together with Brahma complex in organisation of Pol II pausing at promoters of transcriptionally repressed genes;
(iii) to investigate the role of SAYP together with Brahma complex in organisation of Pol II pausing at transcriptionally active genes.

Results Obtained:

Organise the chromatin barrier ahead of the paused mRNA

On the first stage of the project we demonstrated the interaction of SAYP with DHR3 nuclear receptor of ecdysone cascade and characterised their interaction. We demonstrated that DHR3 together with SAYP is essential for transcription of SAYP-dependent genes. They both participate in recruitment of the BTFly (Brahma and TFIID) supercomplex to promoter of the gene. Next we demonstrated that SAYP together with Brahma complex are essential for organisation of Pol II pausing at promoters of transcriptionally repressed genes and organise the chromatin barrier ahead of the paused mRNA. On the next stage we investigated the role of SAYP and Brahma complex in Pol II pausing during active transcription. We demonstrated that at the active transcription stage, SAYP is essential for Ser2 phosphorylation and transcription elongation.

Results Unforeseen in the Original Project:

Pol II pausing is essential for repressed and activated state of genes. At present, it is demonstrated that transcription of the most part of the metazoan genes undergoes Pol II pausing. Our study enlarges the knowledge on Pol II pausing and its molecular mechanism.

Publications:

Vorobyeva, N.E., Nikolenko, J.V., Nabirochkina, E.N., Krasnov, A.N., Shidlovskii, Y.V., Georgieva, S.G. SAYP and Brahma are important for repressive and transient Pol II pausing**.** 2012. Nucleic Acids Res. **40**, 7319-7331

SERBIA

Title: Characterisation of spectinomycin induced chromosomal inversion in *Lactococus lactis* subsp. *lactis* bv. diacetylactis S50

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ICGEB Contract No.: CRP/10/016
ICGEB Reference No.: CRP/YUG10-01

Abstract: *L. lactis* subsp. *lactis* bv. diacetylactis S50 is a producer of proteinase PI type and bacteriocin S50, used as starter culture in dairy industry. It harbours five plasmids ranging from 6 to 140 kb. Spectinomycin resistant colonies of strain S50 were isolated at high frequency. A large chromosomal inversion that is correlated with resistance to high concentration of antibiotic spectinomycin was identified by PFGE. The same type of inversion was identified in four independent experiments and in four different derivatives of strain S50, indicating that the same elements and the same mechanism of recombination were involved as response to antibiotic selective pressure in all used derivatives. Two approaches were applied in order to determine endpoints of inversion: construction of cosmid libraries and expression of spectinomycin resistance in heterologous host and whole genome sequencing of WT strain and invertant and perform comparative analysis of their genomes. None of the methods gave satisfactory results, probably because this is a specific type of resistance that emerged as a result of recombination events that occurred between two homologous sequences located in the regions between two contigs.

Objectives:

The main objective of the project was the determination of endpoints of inversion responsible for induction of spectinomycin resistance in strain S50. Intermediate steps to pursue the original goal are:

(i) Construction of *E. coli*-lactococci shuttle cloning and cosmid vectors as tools for cloning;
(ii) Construction of cosmid libraries of WT strain S50 and its spectinomycin resistant invertant SMS1;
(iii) Transfer of cosmid library and expression of spectinomycin resistance in heterologous lactococcal host;
(iv) Whole genome sequencing, assembling of genomes of S50 and SMS1, comparative analysis of genomes sequences S50 and SMS1, closing of gaps between contigs, detection of endpoints of inversion.

Results Obtained:

In order to determine the endpoints of inversion we followed two main approaches: construction of cosmid libraries and expression of spectinomycin resistance in a heterologous host and whole genome sequencing of WT strain and invertant and comparable analysis of their genomes.

For the purpose of cloning and expression of spectinomycin resistance (developed in strain SMS1 due to occurrence of specific inversion of chromosome) by construction cosmid library of strain S50 and its spectinomycin resistant invertant SMS1, we successfully constructed novel, stable and useful shuttle *E. coli*-lactic acid bacteria cloning (pAZIL) and cosmid (pAZILcos) vectors. Vectors were completely sequenced and theirs stability and efficiency were proved. Using pAZILcos vector, total DNAs of strain S50 and SMS1 digested with *Eco*RI restriction enzyme and STRATAGENE packaging kit, cosmid libraries of both strains were successfully constructed in *E. coli* HB101 cells. Both libraries were obtained with frequency $1.3x10^3$ clones. This is first report of construction of a cosmid library of lactococcal strains. According to determined sizes of lactococcal genomes of ≅2,300 kb we could conclude that obtained frequency of cosmid clones cover complete genomes. Representative number of cosmids (284 that cover lactococall genome at least in duplicate) were transferred from *E. coli* to *L. lactis* subsp. *lactis* IL1403. Transformants with each cosmid were selected for resistance to spectinomycin in order to isolate endpoints of inversion responsible for resistance. Unfortunately, no colonies of IL1403 transformants were able to grow on GM17 containing antibiotic spectinomycin. We suspect that the reason for the absence of resistant colonies could be inability of expression of this specific type of spectinomycin resistance in heterologous host or that sensitivity to antibiotic is dominant in heterozygote.

In order to obtain a reliable complete sequence and to facilitate making orders of contigs of WT strain and invertant two sequencing platforms were applied GS FLX titanium emPCR+Running ¼ Region and GS FLX Titanium Mate Paired end Library Construction (8kb-20kb). Using bioinformatics programs and obtaining whole genome sequencing results, genomes of strains S50 and its derivative SMS1 were assembled. Unfortunately, comparative analysis of genomes did not reveal inverted region in chromosome of invertant SMS1, most probably due to recombination event was

occurred between two repeating sequences that are located in regions between two contigs. Reassembling of scaffolds and contigs in genomes by method closing of gaps is in progress.

Results Unforeseen in the Original Project:

After the construction of cloning and cosmid vectors, we tested the efficiency of cloning and stability of cloned fragments in pAZIL shuttle vector. For that purpose we performed cloning experiments of interesting lactococcal genes, like for novel aggregation factor (from strain BGKP1-laboratory collection). Two important genes for probiotic feature of lactic acid bacteria *aggL* (for lactococcal aggregation factor) and *mbpL* (for lactococcl mucin binding protein) were cloned for the first time and expressed in different lactococcal strains and enterococci.

Publications:

Kojic, M., Jovcic, B., Strahinic, I., Begovic, J., Lozo, J., Veljovic, K., Topisirovic, L. Cloning and expression of a novel lactococcal aggregation factor from *Lactococcus lactis* subsp. *lactis* BGKP1. 2011. BMC Microbiology **11**, 265

SLOVAKIA

Title: Tau truncation: the self-renewing propagator of neurofibrillary degeneration in Alzheimer's disease

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Abstract: Neurofibrillary degeneration caused by misfolded neuronal protein tau is the key pathological feature of Alzheimer's disease. Hyperphosphorylation and truncation are the main posttranslational modifications that distinguish tau in AD from that in normal human brain tissue and thus are the suspected inducers of tau misfolding process.

By immunoproteomic approaches we have characterised a number of truncated tau species in the AD brain tissues in misfolded tau fraction. Their truncation points appear to span C-terminal two thirds of the tau molecule. We showed that tau truncation is not a random process, but a highly specific proteolytic process. Additional post-translational modifications cause systematic shift and clustering of protein bands on SDS gels.

Similar truncation points of tau were found in the misfolded tau in a rat model of tauopathies, which originate from both transgenic and endogenous tau. This confirms that truncated tau can start a self-perpetuating cycle of further tau protein truncation, misfolding and formation of neurofibrillary pathology.

We showed that truncated tau species are also generated in the healthy human brains and normal rats. These "physiological" truncated tau molecules are cleaved in the C-terminal third of tau and arise from distinct proteolytic events.

Objectives:

(i) Identification of novel, pathological forms of tau linked to the individual stages of neurofibrillary development in a rat model of tauopathies; collection of brain samples from transgenic animals for proteomic analysis;

(ii) Purification and proteomic characterisation of the novel, pathological forms of tau protein;

(iii) Characterisation of biological and biochemical properties of the novel truncated tau proteins in cellular models and validation the identified novel tau species of the transformation cascade in human AD samples.

Results Obtained:

We have set up an optimised procedure for insoluble tau proteomic analysis, employing the tandem immunoaffinity chromatography (TIC) procedure coupled to mass spectrometric characterisation.

The analysis of insoluble, misfolded tau species from human AD brains showed that truncation of aggregated tau protein occurs almost exclusively from the N-terminus, which is in agreement with the fact that the C-terminal microtubule binding repeat regions are the aggregation prone domains. N-terminal tau-antibody purified only full-length tau proteins, the so-called A68 tripplet, characteristic of AD pathology, no fragments, only oligomerised species. Tau proteins captured by the C-terminal tau antibody represent a family of N-terminally truncated tau proteins that appear to group into distinct protein bands. This suggests that N-terminal tau protein truncation is a regulated process with several preferential cleavage sites within the tau molecule. In addition, truncated tau proteins are subjected to posttranslational modifications that cause significant and systematic shift in their electrophoretic mobility (e.g., ubiqutination) resulting in groups of protein bands on SDS gels. These data implicate that N-terminal truncation of tau protein in human AD brain is not a random process.

The remaining tau proteins lack both N- and C-termini. They represent a family with wide spectrum of molecular sizes ranging from 10 kDa to high molecular weight aggregates with several distinct bands. Mass spectrometry analysis of the truncated tau proteins identified novel tau truncation points. Some of them are identical to those found in the rat brain samples analysed by a similar method, which on one hand validates that the rat model faithfully reproduces (at the molecular level!) the pathological process of tau misfolding in human AD, and on the other also confirms that the identified tau truncation points in human samples are not resulting from nonspecific cleavage induced by tissue proteases during the long postmortem delay.

By analysing normal human brain we were first to show that tau undergoes "physiological" proteolytic modification – truncation. Western blot analysis of immunopurified tau proteins revealed that truncation generates tau fragments of various sizes with intact N-terminus and a prominent small fragment with intact C-terminus. The N-terminal tau fragments are heterogeneous, since they arose from 6 tau isoforms that are also posttranslationally modified, especially phosphorylated. The C-terminal fragment appears homogeneous simply because this region of tau is identical in all 6 isoforms.

The two groups of soluble truncated tau proteins form a complementary pair, which appears to arise from a single cleavage event. Interestingly, the physiological truncated tau fragments are similar in the human and rat brains.
Our data show that not all tau truncation is detrimental to neurons. The physiological truncation presented here, which takes place in the third repeat region of tau, might even protect tau from misfolding. These results were published in Zilka et al. 2012 (The self-perpetuating tau truncation circle. Biochem. Soc. Trans 40, 681-686).
The presence of many cleavage points of the pathological truncated tau species in the proline-rich and oligomerisation regions, which have on one hand important physiological functions in the binding to microtubules and on the other pathological role in tau misfolding, prompted us to closely characterise structural properties of this part of tau molecule. We prepared cocrystals of Fab fragments of monoclonal antibodies binding to these regions with peptides spanning the epitopes. Crystallisation and preliminary x-ray data analysis was published in Cehlar et al., 2012 (Acta Crystall. section F, 68, 1181-1185).
The selected proteolytic tau fragments identified in the insoluble fraction of the brain homogenate were analysed in *in vitro* tau-tau interaction assay to evaluate their oligomerisation activities. Since the amount of the truncated tau proteins is too low for such a direct functional characterisation, we prepared their recombinant forms (truncated recombinant tau proteins). It appeared that pathological truncated tau proteins show abnormally high oligomerisation activity when compared to normal (recombinant) tau protein. This confirms that they have the potency to induce oligomerisation *in vivo* in brain neurons.
In order to characterise the biological activity of these proteins we expressed them in human neuroblastoma cells. Our experiments thus showed that, unlike normal recombinant tau protein, the truncated tau proteins identified in insoluble, misfolded tau deposits in AD brains are toxic to the neuronal-like cells.
In summary, all these observations support our starting hypothesis that abnormal truncation of tau (e.g., generated under tress conditions) leads to the formation of toxic molecules. Such toxic tau fragments on one hand promote abnormal oligomerisation of tau proteins, and on the other they are directly toxic to the cells, impair their function and lead to death. Thus, they fulfil the requirements for an inducer of neurofibrillary degeneration–toxicity and oligomerisation.

Results Unforeseen in the Original Project:

Interestingly, transfection of the truncated tau constructs into neuroblastoma cells showed that truncated tau becomes abnormally distributed in the cells. Normal, physiological tau proteins are strictly localised to the cytoplasm and only under stress conditions enter the nucleus (Sultan et al., 2011). However, truncated tau proteins were found in the nucleus even without stress, they bypass normal subcellular sorting mechanism. Our experiments showed that the mis-localisation of truncated tau is not the results of the decrease in the molecular size and that the subcellular distribution regulatory region lies within the N-terminal third of the tau protein. Phosphorylation status of tau, the major regulatory mechanism for tau employed by neurons, does not seem to play any role in this process. These findings are submitted for publication.

Publications:

Cehlar, O., Skrabana, R., Kovac, A., Kovacech, B., Novak, M. Crystallisation and preliminary X-ray diffraction analysis of tau protein microtubule-binding motifs in complex with Tau5 and DC25 antibody Fab fragments. 2012. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. **68(Pt 10)**, 1181-1185

Zilka, N., Kovacech, B., Barath, P., Kontsekova, E., Novak, M. The self-perpetuating tau truncation circle. 2012. Biochem. Soc. Trans. **40**, 681-686

Flachbartová, Z., Kovacech, B. Mortalin - a multipotent chaperone regulating cellular processes ranging from viral infection to neurodegeneration. 2013. Acta Virol. **57(1)**, 3-15

SRI LANKA

Title: Characterisation and transfer of Drought Responsive Elements Binding (DREB) genes to rice and evaluation of their effects on drought tolerance

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ICGEB Contract No.: CRP/10/018
ICGEB Reference No.: CRP/SRI10-02

Abstract: Drought and extreme temperature are crucial abiotic stresses, which pose serious impacts upon growth and development of plants and sustainable crop productivity. Continuous efforts in improvement of rice against drought stress using both conventional and molecular breeding techniques need to be carried out to meet the future rice demand. DREB proteins are transcription factors belonging to the AP2/ERF domain containing gene family, which mediates abiotic stress responsive cellular signal transduction.

The main objective of this study was to determine the effect of the expression of OsDREB2A, the DREB2A homolog of indica rice, under the direction of three promoters with the intention of developing drought resistance in a local rice variety. The selected promoters are drought inducible HVA1 promoter of late embryogenesis abundant OsLEA3 gene, OsR40C1 promoter of salinity responsive root specific OsR40C1 gene and the constitutive promoter of CaMV35S. The transgenes were designed to express the OsDREB2A under the control these promoters.

Agrobacterium mediated transfer of transgene constructs resulted in the transformation of calli cells from indica rice strain Bg94-1. Transgenic plants, constitutively and inducibly expressing OsDREB2A were regenerated and positive plants were selected. Gene expression studies and phenotype evaluations will be conducted to study the effect of transgene integration.

Objectives:

The overall objective was to develop drought resistant transgenic rice from local rice varieties by expressing OsDREB2A transcription factor under the control of a drought inducible promoter.

In order to achieve the overall objective the following short-term goals were identified:

(i) To clone and characterise the HVA1-like and Osr40C1 promoters and OsDREB2A coding region;
(ii) To evaluate the promoter activity of HVA1-like promoter and Osr40C1 promoter;
(iii) To study the expression of OsDREB2A gene under the control of three different promoters;
(iv) To study the stress inducible expression of indica OsDREB2A against drought stress.

Results Obtained:

Literature survey and bioinformatics search was carried out to select the candidate gene and promoters. indica OsDREB2A sequence was identified in a homology search using *Arabidopsis* DREB2A and OsDREB2A *Japonica* sequence from NCBI Database. The OsDREB2A *Indica* gene sequence was analysed for its DNA binding domain, localisation signals and protein structure using bioinformatics tools such as BLAST, PREDICT PROTEIN and NCBI structure prediction.

To isolate OsDREB2A, coding region DNA was extracted from drought resistant INGER46 variety and was PCR amplified using restriction sites adapted primers followed by cloning in to pUC18 cloning vector. The OsR40C1 promoter and the HVA1-like promoter were amplified from Pokkali and INGER46 varieties respectively and cloned in to pUC18. The OsDREB2A gene, the HVA1-like promoter and the OsR40C1 promoter in pUC18, were amplified and sequenced for sequence analysis and characterisation. The HVA1 and OsR40C1 promoters were cloned in front of the GUS reporter gene in the pCAMBIA1391 binary vector. These transgene constructs were designed for assessing drought-induced activity of the two promoters. The pCAMBIA1391 vector harbouring transgene construct HVA1-GUS, OsR40C-GUS and CaMV35S-GUS gene construct in pCAMBIA1301 were initially transformed in to *E-Coli* for storage and subsequently mobilised into *Agrobacterium tumefacience* strain GV3101.

The transgenes designed to express the OsDREB2A under the control of drought inducible HVA1 like (HVA1-OsDREB2A), salinity inducible OsR40C1 (OsR40C1-OsDREB2A) and CaMV35S (CaMV35S-OsDREB2A) were constructed on pUC18 backbone and subsequently cloned upstream of NoS terminator of pCAMBIA1390.

Agrobacterium tumefaciens strain GV3101, carrying binary plasmid vectors containing respective transgene constructs, has been employed to transform calli cells from indica rice strain Bg94-1. Calli infected with respective Promoter-Gus constructs and Promoter-OsDREB2A constructs were grown in callus induction medium and selection of the transformed calli from the non-transformed was done with respective antibiotics.

Regeneration of intact plants was done under respective shoot and root regeneration hormone conditions. Intact plants were acclimatised under greenhouse conditions. Transgenic plants expressing HVA1 and CaMV35S promoters were grown and positive transformants were selected using PCR and GuS analysis. GUS histochemical analysis was done for calli expressing GUS

reporter gene regulated by HVA1 and CaMV35S promoter under different exogenous stresses to study the inducible activity of promoters.
Transgenic plants, constitutively and inducibly expressing OsDREB2A were regenerated and positive plants were selected. Gene expression studies and phenotype evaluations will be conducted to study the effect of transgene integration.

Publications:

De Silva, W.S.I., Jayasekera, G.A.U. Fernandopulle, N.D., Hettiarachchi, C. Identification, cloning and *in-silico* characterisation of drought inducible OsDREB2A transcription factor from Indica rice varieties. 2014. Intl. J. of Adv. Biotec. and Res. (in press)

SUDAN

Title: Genetic epidemiology of common cancers in East Africa
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ICGEB Contract No.: CRP/10/019
ICGEB Reference No.: CRP/SUD10-01
Abstract: The increase of cancer incidence in East Africa could be attributed to several factors including common and population specific genetic variants, epigenetic events and viruses within a model of gene environment interaction. The objectives of this study were to determine (i) the prevalence of glutathione gene polymorphisms GSTM1 and GSTT1 among breast cancer patients; (ii) the role of epigenetic modification in breast cancer (for this specific goal we focused on DNA methyltransferases (Dnmt3b) mutations and methylome portfolio of breast cancer tissue as compared to normal tissues using Illumina Human methylation 450); (iii) the role of Epstein-Barr viruses (EBV) in breast cancer and the possible interaction with epigenetic silencing in breast cancer by identifying the effect of EBV-LMP2A on Dnmt3b expression and the down regulatory effect of Dnmt3b on TET1. The last objective of this research aimed to determine why some individuals tend to develop cancer while others do not by looking at p53 Arg/Pro polymorphism.
Results Obtained:
GSTM1 null allele was more frequent among breast cancer Sudanese patients: 27% versus 7.5% in controls (OR: 4.6, 95% CI: 1.9-, 11.5, p value = 0.0007). The methylome results showed 13,633 hypermethylated sites corresponding to 5,339 genes, and 6,555 hypomethylated sites corresponding to 2,811 genes. We looked for potential breast cancer biomarkers and we detected 12 different genes. In particular the SND1 gene, which functions as a transcriptional co-activator for the Epstein-Barr virus nuclear antigen; another gene, HERC5, is responsible for antiviral immunity, while yet another gene, PGLYRP2, is also implicated in innate immune response. These results hint at a possible role for the Epstein-Barr virus in breast cancer biology.
Differential analysis identified hypomethylated promoters for six different miRNAs, of these miR153-2 was of particular interest as linked to a myriad of epithelial cancers. The prevalence of EBV in Sudanese breast cancer samples was found 100% by in situ hybridisation and 54% by PCR while 14% in Eritrean breast cancer samples by PCR. The Arg allele has been shown to pose a major risk in breast cancer (OR = 13). Sequence analysis of 898bp of the DNMT3b gene in Sudanese breast cancer samples revealed the presence of an intronic novel polymorphism and a reported polymorphism. More breast cancer samples from both Sudan and Eritrea were genotyped for the C/T polymorphism (rs2424913): a total of 177 samples from Sudan (88 cases and 89 controls) and 106 (71 cases and 35 controls) from Eritrea were included in the study. C allele was found to be a risk factor for the breast cancer disease (X^2= 9.209, p= 0.0024) in Sudanese samples and no association was detected in Eritrean samples (X^2= 0.303, p= 0.5821).
The expression profile (using immunohistochemistry) of Dnmt3b was detected in 85% of breast cancer tissue while none of the tissue showed expression of TET1. We failed to detect EBV-LAMP2 in theses samples, which we attributed to technical problems. The study also highlighted the role of EBV virus and epigenetic modification in breast cancer.
Publications:
Eltahir, H.A., Adam, A.A., Yahia, Z.A., Ali, N.F., Mursi, D.M., Higazi, A.M., Eid, N.A., Elhassan, A.M., Mohamed, H.S., Ibrahim, M.E. p53 codon 72 arginine/proline polymorphism and cancer in Sudan. 2012. Mol. Biol. Rep. 39(12), 10833-10836
Eltahir, H.A., Elhassan, A.M., Ibrahim, M.E. Contribution of retinoblastoma LOH and the p53 Arg/Pro polymorphism to cervical cancer. 2012. Mol. Med. Rep. **6(3)**, 473-476
Yahia, Z.A., Adam, A.A., Elgizouli, M., Hussein, A., Masri, M.A., Kamal, M., Mohamed, H.S., Alzaki, K., Elhassan, A.M., Hamad, K., Ibrahim, M.E. Epstein Barr virus: a prime candidate of breast cancer aetiology in Sudanese patients. 2014. Infect. Agent Cancer **9(1)**, 9

TUNISIA

Title: Development of a novel vectored vaccine against Hepatitis E using Adeno-Associated Virus expressing truncated HEV capsid protein

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ICGEB Reference No.: CRP/TUN09-01

Objectives:

Hepatitis E infection caused by the hepatitis E virus (HEV) is a common cause of acute hepatitis in developing countries with poor sanitation and hygiene. It is the major cause of acute hepatitis in Southeast and Central Asia and the second most important cause in the Middle East and North Africa. The virus is classified into four genotypes (1-4), with one serotype. Genotypes 1 and 2 exclusively infect human, whereas genotypes 3 and 4 also infect other animals, particularly pigs. HEV is transmitted via an oral-faecal route and affects predominantly young adults; the mortality rate is very high, up to 15-20% in infected pregnant women.

In developed countries, most cases have been imported. However, hepatitis E cases among non-travellers are being increasingly reported in developed countries. Zoonosis is emerging as an important transmission mode.

Currently, no specific treatment for HEV infection is available. The inability to reproducibly culture HEV makes it impossible to develop traditional live or inactivated vaccines.

However, significant progress has been made in developing and testing recombinant subunit vaccines based on the viral capsid protein.

The HEV genome contains three open-reading frames (ORFs) that encode the ORF1 nonstructural polyprotein with proposed biochemical functions, the ORF2 major viral capsid protein, and the ORF3 regulatory protein. Various recombinant forms of the ORF2, expressed using different heterologous systems are being explored.

Several recombinant proteins corresponding to the HEV capsid protein have been shown to induce specific antibodies in animals and to protect against liver injury following subsequent challenge with the virus. In addition an HEV DNA vaccine has also been shown to induce serum anti HEV production in cynomolgus macaques and to protect against re-challenge with heterologous HEV strain.

Among the candidate vaccines against hepatitis E under development two vaccines have undergone clinical studies. A truncated HEV ORF2 protein of 56 kDa produced from a recombinant baculovirus that forms virus like particles; this vaccine has undergone safety and efficacy studies in humans, the recombinant protein was given in an alum-adjuvant formulation and induced the production of anti-HEV among healthy volunteers in a dose-dependent manner. Despite promising results, further studies are needed on the safety of this vaccine for pregnant women, children and certain other groups. This vaccine has not yet reached the market.

The second promising HEV vaccine is prepared by Chinese group, named HEV239 vaccine. It contained a truncated HEV capsid protein, corresponding to amino acids 376-606 expressed in *Escherichia coli* that has been purified and adsorbed on aluminum hydroxide suspended in buffer saline. This vaccine was successfully evaluated in Phase II/III trials, phase III clinical trial published in 2010 showed that it was highly effective in preventing infection among almost 100,000 healthy participants. The vaccine was approved by the China's State Food and Drug Administration (SFDA) in December 2011.

However both vaccines are adjuvanted, delivered intramuscularly and need several injections to be effective. Therefore these vaccines may have a high cost, and cannot be affordable for populations living in developing countries, where HEV is endemic.

Therefore, in this project we suggest to use adeno-associated virus as a vector to deliver the sequence coding for HEVORF2 112-660 amino acids. The vaccine will be given via the nasal route as a less invasive mean; rAAV will be produced by the baculovirus vectors technology.

Results Obtained:

Stocks of recombinant Baculoviruses that encode the capsid proteins (Bac Cap) and the replication proteins (BacRep) of AAV serotype 2 were prepared in Sf9 cells according to standard protocols. To test other serotypes of AAV, we selected AAV5 and AAV6 because of their ability to infect airway epithelial cells, since we aim to deliver the HEV vaccine via the nasal route. In addition, it has been reported that up to 80% of all humans are seropositive for AAV2 and that a significant portion among them carry neutralizing antibodies against AAV2, which may prevent an efficient AAV2 based therapy. Different AAV serotypes may overcome these limitations due to their ability to evade the immune response established against AAV2. Therefore construction of recombinant

baculoviruses were performed and viral stocks of BacRep, BacCap for serotypes 5 & 6 were amplified in Sf9 cells.
In addition, because of the genetic instability of Bac-Rep construct upon serial passages, limiting the expansion to passage 3, and hindering amplification of Bac-Rep stocks for large-scale rAAV production, we used the AAV Rep gene that was genetically modified to express AAV Rep78 and Rep52 polypeptides from a single open reading frame transcriptionally regulated by the polyhedrin promoter, thus allowing expression of the AAV Rep and Cap proteins from the same recombinant baculovirus genome. The resulting Rep/Bac baculovirus was shown to maintain expression of the VP and Rep proteins through at least six amplification passages. Hence Rep/Cap baculovirus vector encoding for the Rep and Cap proteins of AAV2 and 6 were constructed, stocks were also prepared and titrated.
Recombinant baculoviruses were titrated using different methods: $TCID_{50}$ using Sf-9 easy titer cell line, MTT viability test and flow cytometry. All stocks of recombinant baculoviruses showed a titer higher than10^8 pfu/ml as determined by the MTT viability assay.
The recombinant baculovirus coding for the truncated capsid protein of HEV (BacITRHEVORF2) was also constructed, virus titer at the third passage was equal to $6x10^8$ pfu/ml as determined by the MTT viability test. Transduction of HEK 293 EBNA cells with rAAV was carried out; the production of HEVORF2 was confirmed by western blot.
To optimize the production of rAAV of different serotypes in Sf9 cells, we analysed using the experimental design approach the effects of the following factors: initial cell density, time of infection, temperature and individual Multiplicity of infection (MOI) of the three or dual (rAAVT6 and rAAVT2) baculoviruses.
We determined optimal production conditions for all the serotypes of rAAV (2, 5 and 6). We showed that cell density level had a positive effect on rAAV5 and rAAVT6 production. In addition for rAAV2, rAAV2T2 and rAAV5, Sf9 cell infection at 30°C resulted in a higher titer of rAAV compared to 27°C. The highest titer of rAAV was varying between $3.80x10^8$ vg/ml for rAAV2 to $1.04x10^{10}$ vg/ml for rAAVT6.
We have also studied the purification of rAAV using affinity chromatography and cation exchange chromatography. AVB Sepharose affinity medium showed the best yield and an acceptable level of rAAV purity. The recovery yield was equal to 45% for rAAV5; around 100 % for rAAV6 and about 31% for rAAV2. Cation exchange purification resulted in high recovery for rAAV2T2 and rAAV6T2, the yield was around 75 and 100%, respectively.
Currently *in vivo* studies in mice to study the immune response of the candidate vaccine using the intramuscular and nasal routes will start shortly.

Results Unforeseen in the Original Project:

Delay in the execution of the initial program due to several reasons: (i) recruitment of another Ph.D. student during the second year of the project to replace the one who was initially identified and who had interrupted her thesis at the start of the second year of the project; (ii) in addition consumables supply from abroad had required very long time.

Publications:

Trabelsi, K., Kamen, A., Kallel, H. Development of a vectored vaccine against Hepatitis E virus. 2014. Vaccine (in press)

TURKEY

Title: Identification of novel genes taking part in human puberty via autozygosity mapping
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ICGEB Contract No.: CRP/10/021
ICGEB Reference No.: CRP/TUR10-01
Abstract: The ultimate aim of this project is to gain insight in to biological mechanism in human puberty. During this project, in a family with four affected sisters with Normosmic Idiopathic Hypogonadotropic Hypogonadism we identified a deleterious mutation in the KISS1 gene, which encodes Kisspeptin. Kisspeptin is a neuropeptide that robustly elicits GNRH release from the GNRH neurons in the hypothalamus. Previously mutations in KISS1 receptor (GPR54) have been found in patients with Normosmic Idiopathic Hypogonadotropic Hypogonadism. It is for the first time that a loss of function mutation in KISS1 gene, which encodes the ligand, has been identified. This strongly indicates that Kisspeptin is essential for the initiation of puberty and confirms essential function of kisspeptin signaling for the secretion of GNRH from the hypothalamus in humans. Our report came immediately after a significant mouse Kiss1 knock-out study that challenges the requirement of kisspeptin for mouse reproductive function. Thus our studies in this project cleared the GNRH biology in humans.
Objectives:
The ultimate aim of this study is to unravel a biological mechanism that takes role in the initiation of puberty. Discoveries of both currently known signalling pathways in puberty (*i.e.* kisspeptin and Neurokinin B signalling pathways) were thanks to families with multiple affected siblings investigated with autozygosity mapping. In the great majority of such families, the genetic causes are still waiting to be unravelled, which implies that there are more genes to be discovered.
Publications:
Topaloglu, A.K., Tello, J.A., Kotan, L.D., Ozbek, M.N., Yilmaz, M.B., Erdogan, S., Gurbuz, F., Temiz, F., Millar, R.P., Yuksel, B. Inactivating *KISS1* mutation and hypogonadotropic hypogonadism. 2012. N. Eng. J. Med. **366**, 629-635