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Investigation of protein phosphorylation and protein kinases in prokaryotes

Gyula Orosz

Department of Biotechnology, University of Szeged, Szeged, Hungary

Methylococcus capsulatus (Bath) is a Gram-negative, coccoid, methanotrophic bacterium. For the utilization of methane *M. capsulatus* is able to express two methane monooxigenases (MMO): in the presence of copper ions the particulate MMO (pMMO) and its accessory and transport proteins, responsible for copper uptake, are expressed. In the absence of copper the soluble MMO (sMMO) is expressed. sMMO can oxidize a wide range of compounds, from alkanes, alkenes, ethers and haloalkanes to aromatic and even heterocyclic hydrocarbons (Hakemian et al. 2007). Many biodegradation and biotransformation applications for sMMO are currently being investigated.

Although the existence of protein phosphorylation on S, T and Y residues in prokaryotes was first demonstrated in 1978 (Wang et al. 1978), our knowledge about S, T and Y phosphorylation in prokaryotes is very limited. In this recent work the copper regulation of MMO enzymes is studied by comparing the phospoproteome of two cultures grown under distinct conditions and screening for proteins of which's phosphorylation state changes depending on the available copper.

The comparison of the purified phosphoproteomes on 2D ELFO revealed that two subunits of sMMO (smmoB and smmoC) are phosphorylated proteins and unstable elongation factor (EfTU) is only phosphorylated when the media contains no copper. In case of smmoB and EfTU exact phosphorylation sites (smmoB:ser2, EfTU ser144) were determined by mass spectrometry. After changing potential phosphorylation site on smmoB from ser2 to ala by directed mutagenesis the whole enzyme preserved its full activity and smmoB still remained phosphorylated. Furthermore even smmoB heterologously expressed in *E. coli* proved to be phosphorylated by host protein kinases. In order to identify the protein kines(es) that is(are) responsible for the phosphorylation of smmoB a set of kinase deletion mutants were prepared in *E. coli*. After deletion of all known ser/thr and tyr kinases (*yihE, argK, aceK, etk, wzc, hipA, yeaG, yniA*) *E. coli* still preserved its capability to perform protein phosphorylation and smmoB was still phosphorylated, furthermore the deletion of these kinases hardly affected the protein pattern of the whole phosphoproteome of the host bacterium. Although *E. coli* is one of the most studied organisms, its genome is known and well characterized these results suggest that it still may possesses at least one unknown functional protein kinase that is responsible for the phophorylation of the majority of phosphpoproteins including overxpressed smmoB.

During amino acid starvation bacteria activate stringent control elements that result in adaptation to the amino acid shortage by increased amino acid synthesis, restricted protein translation and intensive protein degradation (Chatterji et al. 2001). In *Methylococcus capsulatus* activation of stringent control cascade results in the activation of smmo operon even in copper rich media (unpublished results). Promoting amino acid starvation in *M. capsulatus* grown in copper rich medium also resulted the phosphorylation of EfTU suggesting that phosphorylation of this protein may restrict protein sinthesys *via* direct or indirect inhibition of the translation.

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Supervisor: Kornél L. Kovács
E-mail: ogyula@gmail.com

A novel genetic approach for Identifying genes involved in abscisic acid regulation

Mary Prathiba Joseph

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Abscisic acid is the main stress response hormone in higher plants. In the past few decades many stress regulatory factors were identified which are involved in ABA dependent stress regulation. In order to understand the complicated regulatory web of ABA signaling the Controlled cDNA Overexpression System have been developed (COS, Papdi et al., 2008). We have transformed the *Arabidopsis* Col-0 wild type plants with the COS library and screened progenies of infiltrated plants for ABA insensitivity in the presence and absence of estradiol in germination assays. Screening one million seeds (aproximately 25,000 transformed seeds), of T1 generation resulted 156 plants, which were selected based on their germination capacity on high concentration ABA supplemented media. By testing of T2 generation, estradiol dependent ABA insensitivity was confirmed in 32 lines. Estradiol dependent ABA insenstitive germination was most notable in A26 and A44 lines, which were able to germinate in the presence of 5μM ABA, which otherwise completely inhibited the germination of wild type seeds. Insertions were identified in both lines and corresponded to full-length cDNA encoding the small heat-shock protein HSP17.6A-cII (A26) and a previously unknown zinc-finger domain containing transcription factor protein (A44). GFP fusion and HA-tagging experiments showed nuclear localization of the A44-derived transcription factor. While constitutive overexpression of this transcription factor reduced

fertility, insertion mutants, where transcription of the corresponding gene was abolished, were hypersensitive to ABA. Our results show, that the COS system is suitable for the identification of novel ABA regulatory factors.

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Supervisor: Laszlo Szabados
Email: mary.prathiba@gmail.com

Expression and epigenetic studies of MDR1 genes in drug-resistant rat cells

Ádám Sike

Department of Biochemistry and Molecular Biology, University of Szeged, Szeged, Hungary

The overexpression of multidrug resistance 1 protein (MDR1, Abcb1 or P-glycoprotein), a member of the ABC (ATP Binding Cassette) transporter superfamily, can be responsible for the decreased efficiency of chemotherapeutic drugs in tumour cells. MDR1 is an energy-dependent transporter that is able to extrude cytotoxic agents from the cell. In the presence of these drugs MDR1 expression is up-regulated by different mechanisms, though the molecular background of increased MDR expression is mostly unknown. Recent studies suggested that epigenetic modifications (e.g. histone acetylation, methylation) might play an important role in this process.

The aim of our study was to reveal epigenetic modifications responsible for the increased MDR1 level in multidrug resistant cell lines.

We studied the MDR expression in drug resistant rat hepatoma cells kindly provided by A. Venetianer. The cell lines we used in our experiments were a drug sensitive parental rat hepatoma cell line (D12), a medium (col500) and a highly (col1000) drug-resistant variant of it, selected using increasing concentrations of colchicine (Pirity 1996).

In contrast to humans, rodents have two MDR1 isoforms: Abcb1a and Abcb1b. First, we determined the expression of these genes and found that the mRNA levels of both Abcb1a and Abcb1b were increased in the drug resistant cell lines compared to the parental D12. A potential reason for the elevated expression of the Abcb1 genes is gene amplification. Indeed, we observed an increase in the copies of the Abcb1 genes in the col1000 cell line, however our data suggested that gene amplification was not the (only) reason for the overexpression of Abcb1 genes in the resistant cells.

Next we studied the possible role of histone acetylation in the increased expression of Abcb1 genes. For this, we treated the cells with histone deacetylase inhibitors (Na-butirate and trichostatin A) to maintain the acetylated state of histones. As a consequence of the treatment, the acetylation of H3 and H4 histones increased. Surprisingly, Abcb1a and Abcb1b genes responded to the treatment in an opposite way: the expression of Abcb1a was decreased, while the expression of Abcb1b was increased in cells treated with histone deacetylase inhibitors. Since acetylation of histone 3 lysin 9 and 14 (H3K9ac and H3K14ac) have been shown to play key roles in the regulation of chromatin structure and function, and are linked to transcriptional activation, next we focused on these modifications in order to determine whether they play a role in the differential expression of Abc1a and b genes. Using chromatin immunoprecipitation we determined the H3K9ac and H3K14ac levels at the transcriptional start sites and at upstream regulatory regions of both genes. We found elevated H3K9 and H3K14 acetylation in the col500 resistant cell line in all tested Abcb1 regions. In contrast with that, the acetylation levels of these histones were comparable in the parental D12 and in the other resistant (col1000) cell lines. After histone deacetylase inhibitor treatment, H3K9 and H3K14 acetylation increased in all tested regions of both genes, contrary that, their expression changed in opposite directions.

Since HDAC inhibitors changed the expression levels of Abcb1 genes, we wondered whether this treatment affected the drug efflux capacity of the cells. To answer this question we compared the accumulation of a fluorescent cytotoxin, a substrate of MDR1, in treated and untreated cells. As expected, we detected an increased efflux activity in the drug resistant col500 and col1000 cells; however, TSA-treatment did not influence significantly this process.

In conclusion, our data suggest that elevated Abcb1 gene expression is not always coupled to histone acetylation changes and conversely, the H3K9 and H3K14 acetylation levels do not necessarily predict the expression level of the Abcb1 genes. Thus, further histone acetylation sites and other histone modifications need to be examined to understand the complex regulation of MDR by mechanisms affecting chromatin structure.

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Supervisor: Imre Boros
E-mail: sike.adam@gmail.com