Metabolic engineering and diabetes therapy

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Our group is involved in several research projects on glycogen metabolism, and its alterations in diabetes and Lafora disease (LD). In addition, a second line of research, related to the first, addresses the discovery and characterisation of compounds with anti-diabetic properties. Studies on glycogen metabolism have allowed the identification of many enzymes and intermediate metabolites involved in the synthesis and degradation of this polysaccharide. However, new factors and processes that participate in glycogen regulation are constantly being discovered. Moreover, data on the mechanisms of control in distinct organs and in diverse physiological conditions are incomplete. The alteration of one of these mechanisms may lead to serious pathologies such as diabetes mellitus and LD. The discovery of compounds that counteract the pathological alterations of glucose metabolism are of potential interest for the treatment of diabetes mellitus. Our group has demonstrated changes in the subcellular organisation of the enzymes that participate in glycogen metabolism, depending on the metabolic state of the cells. These changes in localisation represent an additional control mechanism of enzymatic activity, and are probably regulated by post-translational modifications. In addition, we have reported clear differences between muscle an hepatic glycogen metabolism. These differences are based on the distinct subcellular localisation of the glycogen synthase (GS) isoenzymes. We have recently resolved the 3D structure of the smallest known member of the GS family (*Pyrococcus abyssi* GS). This result has allowed the modelling of mammalian GS structures, a first step prior to the resolution of their 3D structure. Finally, we are currently characterising the molecular targets of an anti-diabetic and anti-obesity compound discovered by our group. Phase I of clinical trials with this compound has recently finished, and Phase II is about to start.

Regulation of hepatic, muscular and neuronal glycogen metabolism. Alterations in pathological conditions: diabetes mellitus and Lafora disease

Identification and functional analysis of the phosphorylation sites in the liver isoform of glycogen synthase

Nine phosphorylation sites, relevant for the activation of the muscle isoenzyme of glycogen synthase (MGS) have been described. A comparative sequence analysis showed that the liver isoenzyme (LGS) has 7 of these potential phosphorylation sites, two close to the N-terminus (named 2 and 2a) and five close to the C-terminus (named 3a,3b,3c, 4 and 5).

To progress on the study of the functional consequences of phosphorylation in these sites, we have generated recombinant adenoviruses with serine to alanine mutations on each of these residues (mimicking the non-phosphorylated state); and combined mutations of the N and/or C-terminal phosphorylation regions. Our results show that LGS mutated in a particular site increases its activation state, in contrast to the results published for MGS, for which none of the single mutations increased the activity. In addition, the combined mutation of this site with other phosphorylatable residues exerts a synergistic effect thereby greatly increasing LGS activation state and inducing the accumulation of glycogen independently of allosteric activation by glucose-6-phosphate (G6P). Finally, our findings with these mutants points to the presence of a hierarchy of phosphorylation in LGS. In addition, to study the relevance of the phosphorylation of these sites *in vivo*, we are performing overexpression experiments in animal models using recombinant adenoviruses.

Furthermore, we have identified three new phosphorylation sites of LGS, which correspond to residues that do not share homology with those described for the muscle isoenzyme, through the combined use of affinity chromatography and MALDI-TOF mass-spectrometry. We are currently generating a whole battery of reagents to functionally characterise the

phosphorylation of these novel sequences. These tools will allow to determine the functional significance of the phosphorylation of the newly identified sites and the characterisation of their involvement in the pathogenesis of metabolic diseases like diabetes mellitus. In this context, we have the first recombinant adenoviruses encoding for mutants in the novel phosphorylation sites identified by our group, and we are analysing the functional consequences of phosphorylation/dephosphorylation in these sites.

Analysis of the involvement of the phosphorylation/dephosphorylation events in the subcellular localisation of LGS

Among the putative phosphorylatable residues of LGS, we analysed the phosphorylation at site 3a, which corresponds to serine 641 of the rat sequence. This residue is modified by glycogen synthase kinase-3, (GSK3) and its dephosphorylation has been related to the inactivation of LGS. We have analysed the subcellular localisation of LGS when it is phosphorylated in this residue and our results show that the unmodified protein localised to the sites of active glycogen synthesis. These findings indicate that modulation of the activation state of LGS is probably the result of a complex number of phosphorylation/ dephosphorylation events which drive to changes in the subcellular localisation of the enzyme.

Study of the mechanisms of intranuclear localisation of the muscle isoform of glycogen synthase and its functional consequences

Previous data from our group showed that the accumulation of MGS in the nucleus is inversely correlated with the intracellular glycogen levels. The nuclear localisation of this protein is not diffuse, and our first objective was is to first determine its possible association with diverse subnuclear structures. In this context, we have shown that MGS co-localises with PML and p80-coilin, thus indicating that this isoform is potentially associated with Cajal bodies (See Figure 1.). In addition, we have reported that the region comprising amino acids 555-633 is crucial for the concentration and nuclear aggregation of MGS.

We are working on the identification of MGS-interacting proteins when this enzyme is located in the nucleus. The results obtained will provide crucial information as to the subnuclear localisation of this protein as well as its putative nuclear function. The experimental approach involves affinity purification of MGS from nuclei in non-denaturing conditions and the identification of MGS-interacting proteins by mass-spectrometry. We have identified two nuclear MGS-binding proteins that are involved in the regulation of transcription and splicing.

Analysis of the evolution of the glycogen enzymes: functional implications

Our metabolic and evolutionary studies on glycogen metabolism show that the isoenzymes involved arose from a pre-chordate common ancestor by gene duplication. Furthermore, the main isoenzymes involved in the metabolism of glycogen co-evolved in vertebrates in a concerted fashion in order to fulfil the metabolic requirements/functions of the tissues in which they are expressed. Therefore, we propose

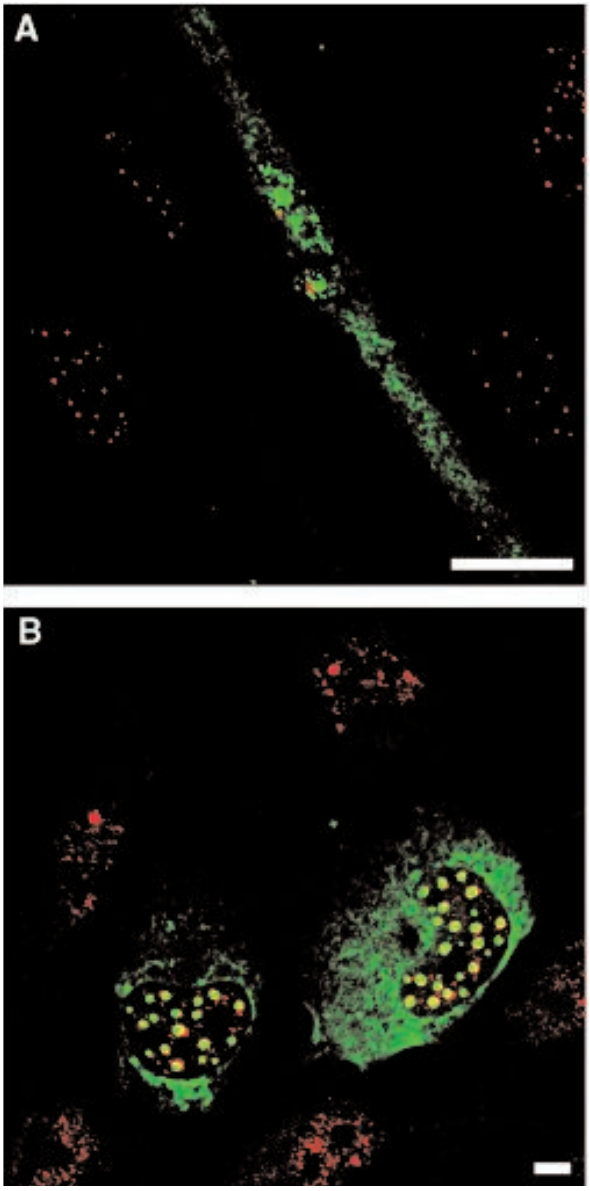

Figure 1*. Co-localisation of HsMGS with markers of subnuclear compartments. (A) Differentiated human myotubes. Cells were subjected to double immunolabelling, to detect MGS (green) and PML (red), respectively. (B) COS-1 cells transiently expressing the GFP-HsMGS fusion protein, in green, immunolabelled with an antibody against p80-coilin, in red. The bar represents 10 µm.*

that tissues are adapted to produce glycogen in their particular metabolic conditions thanks to the concomitant evolution of all the enzymes involved in this process.

The first branch point of glucose metabolism is G6P, whose flux can be diverted either to glycolysis, the pentose phosphate pathway, glycogen synthesis or, in some tissues, back to glucose again. We have shown that the entrance to glycogen metabolism is controlled by the affinity of GS to G6P, rather than physical compartmentalisation of substrates. Moreover, gene duplication allows the independent modulation and evolution of the kinetic features of each GS isoform, thereby allowing liver and muscle to devote glycogen reserves to distinct functions.

Our results show that core metabolic and cellular processes are conserved, but not the mechanisms of control. The addition of control mechanisms enhances the evolvability of the system because they confer flexibility to adapt to new roles. The retroinhibition of high-affinity hexokinases, or GS allosteric activation, both effects mediated by G6P, are clear examples of kinetic features that are modulated through vertebrate evolution to satisfy the diverse roles of glycogen metabolism.

Analysis of the molecular mechanisms involved in the pathogenesis of Lafora Disease

Lafora progressive myoclonus epilepsy (LD, MIM 254780) is an autosomal recessive neurodegenerative disorder characterised by the presence of polyglucosan intracellular inclusion bodies (Lafora bodies), which can be considered aberrant glycogen deposits. Mutations have been identified in two genes, EPM2A (6q24) and EPM2B (6p22.3). The former encodes laforin, a dual-specificity phosphatase with a functional carbohydrate-binding domain. Furthermore, laforin interacts with protein targeting to glycogen (PTG). EPM2B encodes malin, an E3 ubiquitin-ligase that interacts with laforin and promotes its degradation. The physiological roles of laforin and malin are unknown and the cellular processes altered by mutations in these proteins that give rise to the devastating disorder of LD remain to be elucidated. Since Lafora bodies are aberrant molecules of glycogen that accumulate in soma and neural dendrites, we are studying GS activity in neurons and the role of laforin and malin in the regulation of neural glycogen metabolism and, in particular, in the control of GS activity. We have discovered that neurons express MGS (see Figure 2). We are analysing the roles of laforin and malin in the neuronal glycogen metabolism. Our results suggest that laforin has two functions in glycogen synthesis and that it participates in the control of both the activation state and the total levels of key proteins in glycogen synthesis regulation. Taken together, our results indicate that laforin and malin play crucial roles in the maintenance of undetectable or absent levels of glycogen in neuronal cells. In conclusion, laforin and malin are candidates in the control of the intracellular levels of the most important proteins involved in glycogen synthesis, and in preventing the development of glycogen-like intracellular inclusions.

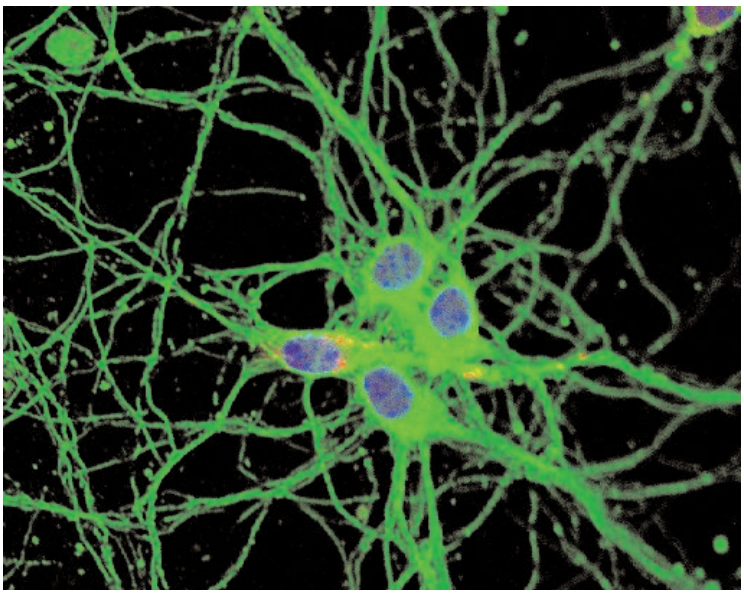

Figure 2. *Immunodetection of MGS in primary cultured neurons. Cells were subjected to double immunolabelling to detect MGS (red) and tubulin (green). Nuclei were stained with Hoechst 33342.*

Analysis of the actions of Lithium ions on glycogen synthase stability

Lithium ions show several remarkable effects on hepatic glycogen metabolism. Thus, LiCl activates LGS in systems such as isolated hepatocytes, primary cultured hepatocytes, and whole animals. This activating action is triggered in the presence and absence of glucose. LGS-induced glycogen synthesis is achieved by both activation and changes in the intracellular location of the enzyme, since effectors that activate GS without causing its translocation fail to stimulate glycogen synthesis. LiCl is a known inhibitor of GSK3 and this property may partly explain the basis of lithium-induced GS activation.

We continued the study of the mechanisms through which LiCl activates LGS and, hence, stimulates hepatic glycogen synthesis. Our results indicate that the LiCl-induced hepatocyte LGS activation is not related to a concomitant translocation of the enzyme. The LiCl action was associated to a significant increase in total GS protein levels with no mod-

ification of GS transcript abundance. Furthermore, the increase in GS levels induced by LiCl was not sensitive to inhibitors of transcription or translation. In contrast, this effect was abrogated by calpain and proteasome inhibitors, suggesting that the mechanism of action of LiCl on the total GS content is related to an increase in the stability of the enzyme.

Dissection of the molecular mechanisms involved in the antidiabetic actions of the oral antidiabetic agent sodium tungstate

Several of our studies describe the anti-diabetic and anti-obesity actions of sodium tungstate when administered orally in animal models of diabetes and obesity. Phase I of clinical trials for this compound has finished and Phase II will start in 2007. Although the metabolic and physiological actions of tungstate have been studied in depth, information on its mechanisms of action is scarce.

Our results show that tungstate exerts its metabolic actions, such as glycogen deposition, amongst others, through a novel mechanism involving the activation of GS and the inactivation of GSK3 through a ras/raf/MEK/ERK/p90RSK dependent-pathway. This process is independent of the insulin receptor, other receptor tyrosine kinases, or the PI3K-PDK1-PKB/Akt transduction pathway branch, the canonical way of inactivating GSK3. The inactivation of this protein kinase contributes to the induction of glycogen deposition stimulated by tungstate. We have used several approaches to attain these results, such as inhibiting several kinases through chemical inhibitors, the use of dominant negative forms of the proteins and expression with adenoviral vectors, among others.

We are currently studying the primary target(s) of tungstate that lead to the downstream events observed: *ie*, activation of ERK and glycogen synthesis deposition. We have preliminary data showing the involvement of PKC and G-proteins in the mechanism of action of tungstate, as well as some data pointing to ionic homeostasis as one of the possible mechanisms modified by treatment with this compound. Furthermore, we have observed inhibition of gluconeogenesis in primary culture hepatocytes when treated with tungstate as compared to untreated cells. Using Real Time PCR and luciferase-coupled assays, we are carrying out several studies on the transcription factors involved in the regulation of the gluconeogenic pathway modified by tungstate.

In addition, we have data indicating that the livers of diabetic animals treated with tungstate show normalisation of the expression of several proteins of the insulin transduction cascade. Moreover, we have also observed a tungstate-dependent normalisation of the state of activation of several components of the insulin cascade, without a modification of total protein amounts.

Our data point to tungstate as an effective anti-diabetic agent and to ERK1/2, PKC and G-proteins as novel targets for the treatment of diabetes.

Crystal structure of glycogen synthase from *Pyrococcus abyssi*: insights into oligomerization and substrate binding of eukaryotic glycogen synthases

The crystal structure of the glycogen synthase from *Pyrococcus abyssi*, the smallest known member of the GS family, revealed that its subunits possess a fold common to other glycosyltransferases a pair of $\beta/\alpha/\beta$ Rossmann fold-type domains with the catalytic site at their interface. Nevertheless, the archaeal enzyme presents an unprecedented homotrimeric molecular arrangement. The C-terminal domains are not involved in intersubunit interactions of the trimeric molecule, thus allowing for movements, likely required for catalysis, across the narrow hinge that connects the N- and C-domains. The radial disposition of the subunits confers on the molecule a distinct triangular shape, clearly visible with negative staining electron microscopy. Comparison of bacterial and eukaryotic glycogen synthases, which use, respectively, ADP or UDP glucose as donor substrates, with the archaeal enzyme, which can utilize both molecules, allowed us to propose the residues that determine glucosyl donor specificity. A structural model of *Pyrococcus abyssi* GS is shown in Figure 3.

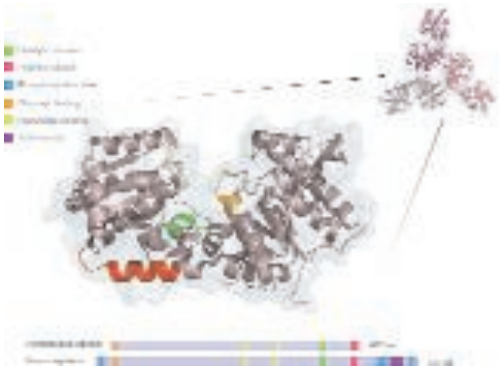

***Figure 3**. 3D structure of* Pyrococcus abyssi *GS showing the similarities shared with Human MGS.*

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RESEARCH NETWORKS AND GRANTS

Mechanisms of regulation and signalling in glycogen metabolism. Molecular analysis and its deffects in diabetes
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Project Coordinator: Joan J Guinovart

Research on the therapeutic targets of the oral antidiabetic agent sodium tungstate
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Regulation of hepatic, muscular and neuronal glycogen metabolism. Alterations on pathological situations: diabetes mellitus and Lafora disease
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Project Coordinator: Joan J Guinovart

OTHER FUNDING SOURCES

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COLLABORATIONS

Characterization of the antidiabetic and antiobesity actions of tungstate
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Determination of the 3D structure of the glycogen synthases
Joan Carles Ferrer (Universitat de Barcelona, Barcelona, Spain)
Ignasi Fita (IRB Barcelona, Spain)

Characterization of glycogen metabolism in reproductive tissue: analysis of alterations in pathological situations
Joan E Rodríguez-Gil (Universitat Autónoma Barcelona, Barcelona, Spain)

Molecular dissection of the mechanisms of action of the antidiabetic agent sodium tungstate in skeletal muscle
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Molecular basis of Lafora Disease
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Phenotypic screening of the molecular targets of the antidiabetic agent tungstate in S. cerevisiae
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Joan J Guinovart's group, March 2006.