



Glycogen metabolism in health and disease

Our group is devoted to the study of the regulatory mechanisms of glycogen metabolism. We focus on the physiological regulation of glycogen deposition and the pathological implications of its alteration. We have a long tradition in the study of glycogen synthase (GS), the key enzyme in the regulation of glycogen synthesis. In order to address relevant biological issues, we combine our knowledge of biochemistry and metabolism with a wide variety of techniques from molecular biology, cell biology, proteomics, RNA silencing, gene transfer, mutant mouse generation, and structural biology. Against the general belief that everything was known about the regulation of glucose metabolism, our discoveries have open new areas of investigation and brought renewed attention to this field. Since altered glycogen deposition may have a causal relation to Lafora disease and other pathologies, we aim to gain a deeper understanding of its regulatory mechanisms and to characterise new therapeutic targets.

Study of glycogen metabolism in diabetes and the search for therapeutic tools

Glycogen synthase (GS) is the only enzyme that catalyses glycogen synthesis. In vertebrates there are two GS isoforms, one expressed exclusively in liver (LGS), and the other expressed in muscle and in most other tissues (MGS). GS expression is elevated in tissues involved in glucose homeostasis and glycogen storage, namely liver, skeletal muscle, heart and even adipose tissue. Liver glycogen metabolism plays a central role in glucose homeostasis and the control of glycemia. In fact, the reduced capacity to accumulate glycogen in the liver of diabetic patients dramatically contributes to hyperglycemia. Thus, LGS activation and stimulation of liver glycogen deposition are potential targets for the treatment of diabetes mellitus.

Analysis of liver glycogen synthase

GS activity is regulated by reversible phosphorylation, in addition to other regulatory mechanisms including allostery, sub-cellular localisation and protein stability. While much research effort has focused on the functional consequences of MGS phosphorylation, little has been devoted to the liver isoform. Nine phosphorylation sites have been described in MGS, and by sequence alignment 7 of these are conserved in LGS. By systematically mutating these 7 serine residues to alanine in the LGS sequence, individually or in pairs, we have identified site 2 (Ser7) as the most potent regulatory site of the activity of the enzyme (Ros *et al.*, 2009). We generated and characterised a constitutively active mutant LGS form. Next we tested the efficacy of increasing LGS activity to improve blood glucose homeostasis in rats in fed and fasted states. The adenovirus-mediated transfer of wild-type LGS to the liver of rats had no effect on blood glucose homeostasis in either state. In contrast, the expression of the active LGS form caused a significant lowering of blood glucose in fed rats but not in fasted ones. Moreover, it markedly

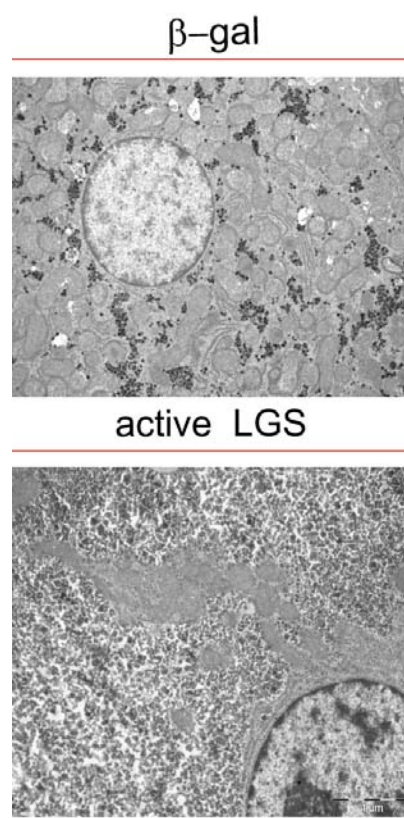
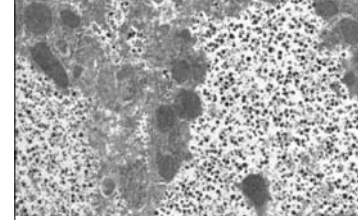


Figure 1. Effects of active LGS overexpression on ultracellular structure as shown by electron microscopy analysis of liver sections. Cellular ultrastructure analysis by electron microscopy of liver biopsies from the rats overexpressing β -gal or the active LGS, fed ad libitum. Note the electron-dense glycogen particles distributed throughout the cytoplasm. The ruler represents 5 nm (taken from Ros *et al.*, in press).



enhanced the clearance of blood glucose when fasted rats were challenged with a glucose load. Hepatic glycogen stores in rats overexpressing active LGS were enhanced in the fed state and in response to an oral glucose load, but showed a net decline during fasting. We conclude that LGS activation improves glucose tolerance in the fed state without compromising glycogenolysis in the post-absorptive state (Ros *et al*, in press). On the basis of these findings, we propose that LGS activation may provide a potential strategy for improving glucose tolerance and normalising glycemia in diabetic states.

In a complementary study, mass spectrometry techniques were used to identify LGS phosphorylation states characteristic of several metabolic conditions. New phosphorylation sites, not conserved in the MGS sequence, were identified in these studies. Next, rat LGS was engineered to mimic phosphorylated species by mutating phosphorylatable serine residues to glutamic acid. Various mutant LGS forms, including single and multiple Ser-to-Glu and Ser-to-Ala substitutions, were generated, subcloned into adenoviral vectors and transferred to primary hepatocytes. We are currently characterising the effects of each mutation on LGS activity, sensitivity to regulation, and subcellular localisation.

Identification of the molecular targets of the anti-diabetic and anti-obesity agent sodium tungstate

Sodium tungstate is an oral glucose-lowering and anti-obesity agent discovered and patented by our group. After demonstration of the activity of this compound in an animal model of type 2 diabetes (ZDF rats), it has completed Phases I and II clinical trials. In collaboration with the groups led by Ramon Gomis (IDIBAPS-Hospital Clínic de Barcelona), Rafael Salto (University of Granada) and Joan Enric Rodríguez Gil (Autonomous University of Barcelona), we have devoted much research effort to unravelling the molecular targets and physiological effects of tungstate. Previous work indicated that this compound induces the activation of the MAP kinase pathway. Our recent experiments show that G-protein activation is involved in the mechanism of tungstate, since Pertussis Toxin, a G-protein inhibitor specific for G_i and G_o , blocks both tungstate-induced ERK phosphorylation and glycogen deposition in primary hepatocytes. These results are being validated by combining tungstate treatment with G protein knock-down (shRNA) or signalling disruption in human and rat cells.

In addition to ERK phosphorylation, we have observed a tungstate-induced normalisa-

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tion of PEPCK expression in treated diabetic rats. This result indicates that tungstate reduces the activation of the gluconeogenic pathway associated with diabetes mellitus, thereby contributing to the lowering of circulating glucose. We extended this research by studying the effects of tungstate on several gluconeogenesis-related genes. We have detected significant changes in the expression of regulatory proteins affecting the control of this pathway.

Study of glycogen metabolism in neurons and the consequences of its deregulation

Although glycogen is present in most cells, its metabolism has been studied mainly in liver and muscle. Nevertheless, there are some cell types, like neurons, that do not accumulate this polysaccharide. We have demonstrated that neurons express GS, specifically MGS. This is a remarkable finding because these cells do not normally accumulate glycogen. However, GS activity in neurons is tightly blocked through previously described regulatory mechanisms (phosphorylation, subcellular localisation) and through a new mechanism that involves the coordinated action of malin and laforin proteins. Mutation or inactivation of these two genes, together representing more than 90% of the genetic defects found in Lafora disease patients, results in the suppression of one control level of glycogen accumulation and in the formation of non-degradable glycogen aggregates. Our results also show that excessive glycogen accumulation in neurons induces apoptosis (Vilchez *et al*, 2007).

The concept that glycogen is harmful for neurons has completely changed our vision of the field. During 2009 we have oriented our research to gaining a better understanding of the following: the physiological role of GS in neurons; the (pathological) conditions that induce glycogen accumulation in neurons; and the apoptotic pathway activated by glycogen accumulation. In addition, we have generated a number of transgenic mouse models of (conditional) gain- and loss-of-function of GS and associated regulatory proteins. Using Cre-recombinase technology, we are able to direct the overexpression or deletion of our gene of interest to specific cell types. These animal models will provide further insight into the physio-pathological implications of abnormal glycogen accumulation *in vivo*.

Study of physiological role of GS expression in neurons

As stated above, neurons express GS but do not normally accumulate glycogen. Furthermore, glycogen accumulation is harmful for neurons. The obvious question is why neurons use energy to express GS and keep it strictly blocked. We are addressing this issue through a range of approaches. First, using immunohistochemistry, we are studying the expression of MGS in the mouse brain at various developmental stages, in order to identify the neurons and stages in which higher levels of MGS are expressed, and whether this increased expression is related to transient periods of tolerated glycogen deposition. Second, we hypothesised that MGS has a moonlighting activity in addition to its function in glycogen synthesis. Since MGS translocates to the nucleus in cells lacking glycogen deposits, we have studied the putative nuclear function of this enzyme. By co-immunoprecipitation techniques, we have identified MGS-binding proteins that participate in RNA processing. We are currently testing the capacity

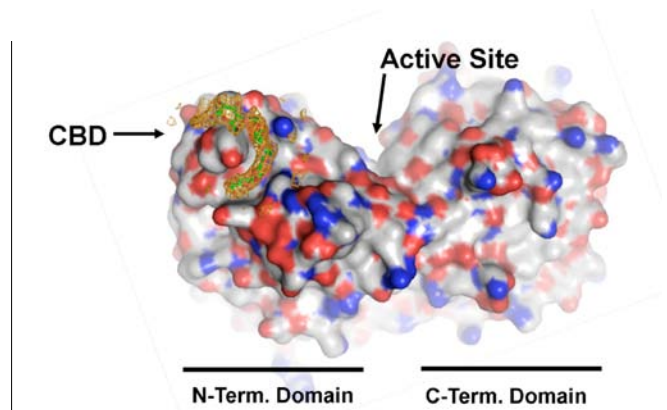


Figure 2. Crystallographic structure of monomeric PaGS. The enzyme is represented by coloured surfaces (carbon atoms in grey, nitrogen in blue and oxygen in red) while the bound maltotetraose molecule is shown by sticks, including its electron density. CBD, Carbohydrate Binding Domain.

of MGS to directly bind RNA. Furthermore, in order to dissect the regulatory events that determine MGS subcellular localisation and proteasome-mediated degradation, we have generated a collection of mutant MGS forms.

Study of the (pathological) conditions that induce glycogen accumulation in neurons

A review of the literature indicates that the presence of glycogen in neurons in certain neurological diseases was reported many years ago. Moreover, the presence of intracellular bodies composed mainly of glucose polymers has been recognised in many pathological conditions. The nomenclature used to describe these structures is varied: polyglucosan bodies, *corpora amylacea* and Lafora bodies, among others. However, all these structures share a common feature, namely they are essentially formed by poorly branched glycogen. Indeed, abnormal glycogen deposits are found not only in Lafora disease patients, but also in those with other syndromes commonly designated as Glycogen Storage Diseases (GSDs) or glycogenoses. Several of these rare diseases have a recessive monogenic origin, involving a number of proteins related to glycogen synthesis and disposal. In addition, polyglucosan bodies can be found as a consequence of prolonged hyperglycemia in diabetic patients.

In collaboration with Ramon Gomis (IDIBAPS-Hospital Clínic de Barcelona) and Rafael Simó (Hospital Vall d'Hebron), and in the context of a CIBERDEM coordinated project, we have studied the presence of abnormal glycogen deposits in pancreatic β -cells, retinal neurons, and nephrons, and the relation of these deposits with the diabetic complications affecting these organs (*ie* retinopathy, nephropathy). We have demonstrated the expression of MGS in these cell types and the accumulation of glycogen in the retina of diabetic patients. In addition, we are generating tissue-specific transgenic mouse models in order to assess whether glycogen accumulation by itself is causative of said pathologies. Finally, we are interested in the potential relation

between hypoxia, aging, glycogen accumulation and neurodegeneration. In this regard, we have used an *in vitro* model to study the effects of hypoxia in neurons. We have observed that under these conditions MGS is strongly activated. We are currently extending these studies, as well as testing human samples obtained from elderly subjects and patients with neurodegenerative diseases.

Study of the apoptotic pathway activated by glycogen accumulation in neurons

In an effort to characterise the apoptotic pathway activated by abnormal glycogen deposition in neurons, we have studied the level of activation of several proteases that drive the cell death programme. First, by expressing a constitutively active GS form, we confirmed that enforced glycogen accumulation induces neuronal apoptosis. Second, we observed that glycogen accumulation induces the activation of Bid, Caspase 8 and Caspase 3, thereby suggesting the activation of the extrinsic apoptosis signalling pathway. Finally, a major effort has been made in the generation of *in vivo* models for the study of glycogen-induced neurodegeneration. In addition to the above-mentioned transgenic mouse models, we have optimised intracranial stereotactic injection for the delivery of viral vectors to specific brain areas in mice. This technique will allow us to force the expression or silencing of genes of interest and thus to study their role in the regulation of neuronal glycogen metabolism and glycogen-induced apoptosis.

Structural approach to the elucidation of the catalytic mechanism and the regulation of GS

The expression and purification of mammalian GS has proven extremely difficult. However, we have been able to purify GS from *Pyrococcus abyssi* (PaGS) and crystallise it as a trimeric complex. In order to reduce the flexibility of the trimeric structure and improve resolution, we have recently generated a monomeric PaGS mutant form, which can be purified and crystallised in the presence of substrates. Crystals obtained in the presence of UDPG and maltohexaose were subjected to X-Ray diffraction to generate a new 2.6 Å resolution structure. Surprisingly, this structure shows the enzyme in an open conformation with no substrate present in the active site. Instead, a glucose polymer is found tightly bound to the surface of the N-terminal domain of the protein (Figure 2). This result offers structural evidence of the existence of a carbohydrate binding domain in the N-terminal part of GS, which most likely facilitates the binding and retention of the enzyme to the growing glycogen particles *in vivo*. Importantly, this carbohydrate binding site is structurally conserved in *Escherichia coli* GS and in glycogen phosphorylase, and a similar domain can be deduced by sequence alignment in mammalian GSs. We are currently studying the functional role of this glycogen binding site in human MGS by generating single amino acid mutant forms and testing their binding affinity to glycogen and their specific activity. FRAP results demonstrate a marked reduction of glycogen binding capacity when conserved tyrosine residues in the carbohydrate binding domain are mutated to alanine.

Scientific output

Publications

Guinovart JJ. Mind the gap: bringing scientists and society together. *Cell*, **137**(5), 793-95 (2009)

Reverter JL, Nadal J, Fernández-Novell JM, Ballester J, Ramió-Lluch L, Rivera MM, Elizalde J, Abengoechea S, Guinovart JJ and Rodríguez-Gil JE. Tyrosine phosphorylation of vitreous inflammatory and angiogenic peptides and proteins in diabetic retinopathy. *Invest Ophthalmol Vis Sci*, **50**(3), 1378-82 (2009)

Ros S, García-Rocha M, Domínguez J, Ferrer JC and Guinovart JJ. Control of liver glycogen synthase activity and intracellular distribution by phosphorylation. *J Biol Chem*, **284**(10), 6370-78 (2009)

Vilchez D, Rodríguez De Córdoba S and Guinovart JJ. Enfermedad de Lafora: epilepsia y regulación el metabolismo del glucógeno por laforina y malina. In Monografía XXV Avances en Neurociencia: Neurotransmisores y Patologías Nerviosas (Miras T, Rodríguez A, ed.), Real Academia Nacional de Farmacia (2009)

Research networks and grants

Actividades Comité IUBMB

Spanish Ministry of Science and Innovation, MEC-IUBMB (2007-2009)
Principal investigator: Joan J Guinovart

Diabetes and obesity treatment by tungstate: metabolic and molecular targets
Carlos III Health Institute, CIBERDEM–DOTUM (2009-2010)

Diabetes y enfermedades metabólicas asociadas (CIBERDEM)
Carlos III Health Institute, CB07-08-0045 (since 2008)
Principal investigator: Joan J Guinovart

Enfermedad de Lafora: papel de laforina y malina
'La Caixa' Foundation (2006-2009)
Principal investigator: Joan J Guinovart

Estudio de un nuevo mecanismo de regulación del metabolismo del glucógeno. Análisis de las implicaciones patológicas de la acumulación anómala de polímeros de glucosa
Spanish Ministry of Science and Innovation, BFU2008-00769 (2009-2011)
Principal investigator: Joan J Guinovart

Glycogen-induced dysfunctions in pancreas and retina and their involvement in the ethiogenesis of diabetes mellitus (GIDIPRED)
Carlos III Health Institute, CIBERDEM–GIDIPRED (2009-2010)

Mejora de la predicción traslacional de los ensayos de seguridad no clínica al hombre
NOSCIRA SA (2007-2010)
Principal investigator: Joan J Guinovart

Molecular basis of progressive myoclonus epilepsy of the Lafora type
'La Marató TV3' Foundation (2007-2009)
Principal investigator: Joan J Guinovart

Nuevos fármacos y dianas para el tratamiento de diabetes mellitus
'Marcelino Botin' Foundation (2006-2010)
Principal investigator: Joan J Guinovart

Collaborations

Analysis of the 3D structure of glycogen synthase

Joan C Ferrer, University of Barcelona (Barcelona, Spain)

Characterization of glycogen metabolism in reproductive tissue: analysis of alterations in pathological situations

Joan E Rodríguez-Gil, Autonomous University of Barcelona (Barcelona, Spain)

Characterization of the anti-diabetic and anti-obesity actions of tungstate

Ramon Gomis, IDIBAPS-Hospital Clínic de Barcelona (Barcelona, Spain)

Determination of the 3D structure of the glycogen synthases

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Glycogen-induced dysfunctions in pancreas and retina and their involvement in the ethiogenesis of diabetes mellitus

Ramon Gomis, IDIBAPS-Hospital Clínic de Barcelona (Barcelona, Spain); Rafael Simó, Institut de Recerca Hospital Vall d'Hebrón (Barcelona, Spain)

Histological analysis of the alterations in the neuronal glycogen metabolism in neurological diseases

Teresa Ribalta, Hospital Clínic de Barcelona (Barcelona, Spain)

In silico design of modulators of the glycogen synthase activity

Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Laser induced forward transfer: a direct writing technique for biosensors preparation

José L Morenza, University of Barcelona (Barcelona, Spain)

Mechanism of action of anti-hyperglycaemic compounds and development of in vitro methods for screening mode of action

Loranne Agius, School of Clinical Medical Sciences-Diabetes, The Medical School (Newcastle, UK)

Molecular basis of Lafora disease

Santiago Rodríguez de Córdoba, Centro de Investigaciones Biológicas, CSIC (Madrid, Spain); Pascual Sanz, Instituto de Biomedicina de Valencia, CSIC (Valencia, Spain)

Molecular dissection of the mechanisms of action of the anti-diabetic agent sodium tungstate in skeletal muscle

Rafael Salto and M^a Dolores Girón, University of Granada (Granada, Spain)

Relation between the diabetic syndrome and the key glucose homeostasis enzymes, fructose-1,6-Biphosphatase and glycogen synthase

Juan Carlos Slebe, Instituto de Bioquímica, Universidad Austral de Chile (Valdivia, Chile)

Study of hypoxia and glycogen accumulation

Luis del Peso, Instituto de Investigaciones Biomédicas, CSIC (Madrid, Spain)

Study of the actions of sodium tungstate on the ionic homeostasis

Miguel A Valverde, Pompeu Fabra University (Barcelona, Spain)

Study of the alterations in glycogen metabolism associated with colon cancer

Santiago Ramón y Cajal, Institut de Recerca Hospital Vall d'Hebrón (Barcelona, Spain)

Study of the alterations of glycogen metabolism in animal models with neurological diseases

Martí Pumarola, Autonomous University of Barcelona (Barcelona, Spain)

Study of the molecular targets and biological actions of sodium tungstate

José Ramón Murguía, Universidad Politécnica de Valencia (Valencia, Spain)

Study of the proteomic alterations induced by tungstate treatment of diabetic animals

Carmen Cámara, Universidad Complutense de Madrid (Madrid, Spain)

The use of Drosophila melanogaster as model system for the study of Lafora disease

Marco Milán, IRB Barcelona (Barcelona, Spain)

Awards and honours

Prat de la Riba award

Institut d'Estudis Catalans (2009)

Awardee: Joan J Guinovart