The International Journal of Biochemistry & Cell Biology 45 (2013) 1479–1488

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Glycogenic activity of R6, a protein phosphatase 1 regulatory subunit, is modulated by the laforin–malin complex

Carla Rubio-Villena, Maria Adelaida Garcia-Gimeno [1], Pascual Sanz [*,1]

Instituto de Biomedicina de Valencia, CSIC, and Centro de Investigación en Red de Enfermedades Raras (CIBERER), Jaime Roig 11, 46010 Valencia, Spain

ARTICLE INFO

Article history:
Received 21 January 2013
Received in revised form 16 April 2013
Accepted 17 April 2013
Available online 26 April 2013

Keywords:
Laforin
Malin
Lafora disease
Protein phosphatase type 1
Glycogen regulation
PPP1R3D

ABSTRACT

Protein phosphatase type 1 (PP1) plays a major role in the regulation of glycogen biosynthesis. PP1 is recruited to sites of glycogen formation by its binding to specific targeting subunits. There, it dephosphorylates different enzymes involved in glycogen homeostasis leading to an activation of glycogen biosynthesis. Regulation of these targeting subunits is crucial, as excess of them leads to an enhancement of the action of PP1, which results in glycogen accumulation. In this work we present evidence that PPP1R3D (R6), one of the PP1 glycogenic targeting subunits, interacts physically with laforin, a glucan phosphatase involved in Lafora disease, a fatal type of progressive myoclonus epilepsy. Binding of R6 to laforin allows the ubiquitination of R6 by the E3-ubiquitin ligase malin, what targets R6 for autophagic degradation. As a result of the action of the laforin–malin complex on R6, its glycogenic activity is downregulated. Since R6 is expressed in brain, our results suggest that the laforin–malin complex downregulates the glycogenic activity of R6 present in neuron cells to prevent glycogen accumulation.
© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Protein phosphatase 1 (PP1) is a serine/threonine protein phosphatase which plays important roles in the regulation of a broad range of physiological processes, including cell proliferation, differentiation, survival and death (Heroes et al., 2012). In the later years it has become evident that free PP1 has essentially no specificity for substrates, its specificity being regulated by more than 200 PP1-interacting proteins (PIPs), which create a vast variety of PP1 holoenzymes with a distinct subset of substrates and mechanisms of regulation (Bollen et al., 2010; Heroes et al., 2012). One of the processes regulated by PP1 is glycogen homeostasis. In this case, PP1 interacts with a specific set of glycogen targeting subunits which contain a carbohydrate binding module of the CBM21 type. Seven recognized glycogen targeting subunits have been identified, namely PPP1R3A (GM), PPP1R3B (GL), PPP1R3C (R5/PTG), PPP1R3D (R6), PPP1R3E (R3E), PPP1R3F (R3F) and PPP1R3G (R3G) (in brackets, the informal name) (Heroes et al., 2012). These subunits target PP1 to specific enzymes involved in glycogen homeostasis: they bind glycogen synthase (GS), allowing in this way the dephosphorylation of the synthase and its activation; they also bind glycogen phosphorylase (Ph) and glycogen phosphorylase kinase (PhK), allowing the dephosphorylation and inactivation of these enzymes. In this way the net action of PP1 on glycogen homeostasis is to improve glycogen accumulation, since it promotes the synthesis of the polysaccharide and prevents its degradation (Roach et al., 2012). Although these subunits bind to PP1 through a similar RVxF docking motif, they are structurally different and more importantly, their function is regulated by different mechanisms depending on the corresponding subunit. For example, the activity of GM is regulated by phosphorylation of the subunit by PKA, whereas the activity of GL is inhibited allosterically by the binding of Ph to the C-terminus of the regulatory subunit (Roach et al., 2012).

The PP1-PPP1R3X holoenzymes play a main role in glucose homeostasis since they regulate glycogen synthesis and degradation in key tissues such as liver and skeletal muscle. In addition, the participation of R5/PTG in the regulation of glycogen synthesis in the neurons has been described recently. In fact, enhancement of R5/PTG function leads to the accumulation of insoluble polyglucosans in neuronal cells (Vilchez et al., 2007), and R5/PTG is associated with a fatal type of progressive myoclonus epilepsy named Lafora disease (LD, OMIM 254780). This disease is produced by mutations in either the EPM2A gene, encoding the glucan phosphatase laforin, or the EPM2B gene, encoding the E3-ubiquitin ligase malin. These two proteins form a functional complex and defects in any of them lead to the disease, which correlates with the appearance of poorly-branched insoluble polyglucosans named Lafora bodies. It has been proposed that the laforin–malin complex interacts with R5/PTG and ubiquitinates it, impairing its glycogenic

Abbreviations: AMPK, AMP-activated protein kinase; GAD, Gal4 activating domain; GS, glycogen synthase; LD, Lafora disease; PKA, protein kinase A; Ph, glycogen phosphorylase.

* Corresponding author. Tel.: +34 963391779; fax: +34 963690800.
E-mail address: sanz@ibv.csic.es (P. Sanz).

[1] These two senior authors contributed equally to this work.

1357-2725/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2013.04.019

activity (Vilchez et al., 2007; Solaz-Fuster et al., 2008; Worby et al., 2008). In addition, we have recently reported that the key metabolic sensor AMP-activated protein kinase (AMPK), on one hand enhances the function of the laforin–malin complex, and on the other hand, it is able to phosphorylate R5/PTG, facilitating in this way the recognition of this subunit by the laforin–malin complex and its ubiquitination (Solaz-Fuster et al., 2008; Vernia et al., 2009).

Very little is known about the mechanism of regulation of R6 (PPP1R3D) activity. Since R6 is expressed in brain (Armstrong et al., 1997; Esteves et al., 2012), this subunit could also have a role in regulating glycogen synthesis in this organ. For this reason we analyze the glycogenic activity of this subunit and whether the activity of R6 could also be regulated by the laforin–malin complex. In this work we present evidence of a physical interaction between laforin and R6. This interaction leads to the malin-dependent ubiquitination of R6, which is targeted for autophagic degradation. Using a neuronal cell model (Neuro-2a), we also show that R6, laforin, malin and glycogen synthase are located in similar cytosolic areas of the cell, revealing a common role of these proteins on the regulation of glycogen homeostasis in this neuronal cell type.

2. Materials and methods

2.1. Plasmids

pCMV-HA-laforin, pcDNA3-HA-malin and pFlag-R6 constructs were described previously (Solaz-Fuster et al., 2008; Garcia-Haro et al., 2010). pBTM-R6 and pGADT7-R6 plasmids were obtained by digesting a R6 plasmid (Worby et al., 2008) with EcoRI/XhoI and subcloning the fragment containing the R6 open reading frame (ORF) into pBTM116 (EcoRI/SalI) or pGADT7 plasmids, respectively. Plasmid pEYFP-R6 was obtained by digesting plasmid pGADT7-R6 with HindIII/KpnI and subcloning the resulting fragment into pEYFP-C1 (BD Biosciences, Madrid, Spain). Plasmid pCMV-myc-R6 was obtained by digesting pGADT7-R6 with SfiI/XhoI and subcloning the resulting fragment into pCMVmyc (BD Biosciences, Madrid, Spain). Other plasmids used in this study were pCMV-6xHisUbiq (from Dr. M. Rodriguez, Proteomics Unit, CIC-BioGUNE, Vizcaya, Spain); pCMV-6xHisUbiq K48R and pCMV-6xHisUbiq K63R (from Dr. Ch. Blattner, Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Karlsruhe, Germany); pCMV-Mdm2 (from Dr. M. Gentry, University of Kentucky, Lexington, KY; Worby et al., 2008); pCMV-6xHisHAUbiq KO was kindly provided by Dr. W. Gu (Institute of Cancer Genetics, Columbia University, New York, USA; Li et al., 2003) and pCFP-laforin was a generous gift from Dr. Romá-Mateo (IBV-CSIC, Valencia, Spain).

2.2. Cell culture, transfection and treatments

Murine neuroblastoma Neuro-2a (N2a) and human embryonic kidney (HEK-293) cells were grown in Dulbecco's modified Eagle's medium (Lonza, Barcelona, Spain), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% of inactivated fetal bovine serum (Invitrogen, Madrid, Spain) in a humidified atmosphere at 37 °C with 5% CO_2. Cells were transfected with 1 μg of each plasmid using X-treme GENE HP transfection reagent (Roche Diagnostics, Barcelona, Spain) according to the manufacturer's instructions. When indicated, 18 h after transfection, cells were treated with MG132 (25 μM), ammonium chloride (20 mM)/leupeptin (100 μM) or lactacystin (5 μM) for 6 h. Cell extracts were prepared using lysis buffer [25 mM Tris HCl at pH 7.4, 15 mM EDTA at pH 8, 50 mM NaF, 0.6 M sucrose, 15 mM 2-mercaptoethanol, 15 mM $Na_2P_2O_7$, 1 mM PMSF, and a complete Mini-EDTA free protease inhibitor mixture (Roche Diagnostics, Barcelona, Spain)]. Cells were lysed by repeated passage through 24Gx5/8″ needle. Twenty micrograms of total protein from the soluble fraction of cell lysates were analyzed by SDS-PAGE and Western blotting using appropriated antibodies: anti-myc, anti-Flag, anti-HA (Sigma–Aldrich, Madrid, Spain); anti-tubulin, anti-Mdm2, anti-LexA (Santa Cruz Biotechnology, Barcelona, Spain).

2.3. Yeast two-hybrid analysis

Yeast THY-AP4 strain [*MATα ura3 leu2 trp1 lexA::lacZ lexA::HIS3 lexA::ADE2*] (Paumi et al., 2007) was transformed with plasmids pBTM-R6 (LexA-R6) and pACT2 (GAD, empty plasmid), pACT2-malin (GAD-malin) or pACT2-laforin (GAD-laforin), or with pGADT7-R6 (GAD-R6) and plasmids pBTM (LexA, empty plasmid), pBTM-malin (LexA-malin) or pEG202-laforin (LexA-laforin). Transformants were grown in selective SC medium, and β-galactosidase activity was assayed in permeabilized cells and expressed in Miller units as in Ludin et al. (1998).

2.4. Co-immunoprecipitation analysis

Neuro-2a cells were transfected with pCMV-HA-laforin and pCMV-myc-R6 or with the corresponding empty plasmids. Cells were scraped on ice in lysis buffer [50 mM Tris–HCl pH 8; 10 mM KCl, 50 mM EDTA; 15% glycerol, 1% nonidet P-40 (NP-40), complete protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain), 1 mM PMSF, 50 mM NaF, 2 mM $NaVO_4$ and 5 mM $Na_2P_2O_7$]. Cell lysis was performed as described above. Cell lysates were then centrifuged at 13,000 × *g* for 15 min at 4 °C. R6-laforin complexes were immunoprecipitated from supernatants (500 μg of total protein) with anti-myc polyclonal antibody (Sigma–Aldrich, Madrid, Spain) and visualized by immunoblotting using anti-myc-HRP or anti-HA-HRP (Sigma–Aldrich, Madrid, Spain) antibodies.

2.5. Study of in vivo ubiquitination of R6

For ubiquitination assays, HEK-293 cells were co-transfected with pCMV-myc-R6, 6xHis-tagged ubiquitin plasmids and, when indicated, with pcDNA3-HA-malin and pCMV-HA-laforin plasmids, using X-treme GENE transfection reagent, according to the manufacturer's instructions (Roche Diagnostics, Barcelona, Spain). After 18 h of transfection, when indicated, cell were treated with MG132 (25 μM) for 6 h. Then cells were lysed and ubiquitinated proteins purified by metal affinity chromatography (Kaiser and Tagwerker, 2005). Bound proteins and clarified extracts were analyzed by immunoblotting with the appropriated antibodies.

2.6. Glycogen determination

Neuro-2a transfected cells were scraped on ice into 30% KOH and then heated at 100 °C for 15 min. Glycogen was measured as described previously (Chan and Exton, 1976) and expressed as μg of glucose per mg of protein.

2.7. Immunofluorescence and confocal microscopy

Neuro-2a cells transfected with the indicated plasmids were grown on 12-well plates containing coverslips. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. For direct fluorescence, cells were washed three times with PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min, in order to visualize the nuclei. Then, cells were washed three times with PBS and mounted on slices using Aqua-Poly/Mount coverslipping medium (Polysciences, Inc. Eppelheim, Germany). For immunofluorescence, cells were permeabilized with

Download English Version:

https://daneshyari.com/en/article/1983689

Download Persian Version:

https://daneshyari.com/article/1983689

Daneshyari.com