Available online at www.sciencedirect.com
SCIENCE DIRECT®
Archives of Biochemistry and Biophysics 435 (2005) 112–124
ABB
www.elsevier.com/locate/yabbi

GNN is a self-glucosylating protein involved in the initiation step of glycogen biosynthesis in *Neurospora crassa*☆

Renato Magalhães de Paula[a], Wayne A. Wilson[c], Héctor Francisco Terenzi[b], Peter J. Roach[c], Maria Célia Bertolini[a,*]

[a] *Instituto de Química, UNESP, Departamento de Bioquímica e Tecnologia Química, 14800-900, Araraquara, SP, Brazil*
[b] *Faculdade de Filosofia, Ciências e Letras, Departamento de Biologia, USP, Ribeirão Preto, SP, Brazil*
[c] *Indiana University School of Medicine, Department of Biochemistry and Molecular Biology, 46202-5122, Indianapolis, IN, USA*

Received 21 October 2004, and in revised form 2 December 2004
Available online 23 December 2004

Abstract

The initiation of glycogen synthesis requires the protein glycogenin, which incorporates glucose residues through a self-glucosylation reaction, and then acts as substrate for chain elongation by glycogen synthase and branching enzyme. Numerous sequences of glycogenin-like proteins are available in the databases but the enzymes from mammalian skeletal muscle and from *Saccharomyces cerevisiae* are the best characterized. We report the isolation of a cDNA from the fungus *Neurospora crassa*, which encodes a protein, GNN, which has properties characteristic of glycogenin. The protein is one of the largest glycogenins but shares several conserved domains common to other family members. Recombinant GNN produced in *Escherichia coli* was able to incorporate glucose in a self-glucosylation reaction, to *trans*-glucosylate exogenous substrates, and to act as substrate for chain elongation by glycogen synthase. Recombinant protein was sensitive to C-terminal proteolysis, leading to stable species of around 31 kDa, which maintained all functional properties. The role of GNN as an initiator of glycogen metabolism was confirmed by its ability to complement the glycogen deficiency of a *S. cerevisiae* strain (*glg1 glg2*) lacking glycogenin and unable to accumulate glycogen. Disruption of the *gnn* gene of *N. crassa* by repeat induced point mutation (RIP) resulted in a strain that was unable to synthesize glycogen, even though the glycogen synthase activity was unchanged. Northern blot analysis showed that the *gnn* gene was induced during vegetative growth and was repressed upon carbon starvation.

Keywords: Glycogen; Glycogenin; Repeat induced point mutation; Gene expression; *Neurospora crassa*; Yeast complementation

Glycogen represents a major storage polysaccharide in a wide range of species and its metabolism is under an intricate regulation by sensing nutrient availability and other environmental conditions. The biosynthesis of glycogen is mediated by the initiator glycogenin (EC 2.4.1.186), glycogen synthase (EC 2.4.1.11), and the branching enzyme (EC 2.4.1.18), that mediate formation of the α-1,4- and α-1,6-glycosidic bonds, characteristic of the glycogen molecule. The role of glycogenin was first studied in mammalian systems and it has been implicated in the initiation step of glucose polymerization, through its self-glucosylation activity [1–4]. Mammalian glycogenin is able to attach glucose to a specific Tyr residue and build an oligosaccharide chain that acts as substrate for elongation by glycogen synthase. Simultaneous action of the branching enzyme leads to the formation of mature branched glycogen molecules.

Because of the availability of more and more complete genome sequences, the number of glycogenin-like proteins in the databases has increased substantially in the last few years. However, most of the biochemical and functional studies have focused on the mammalian and yeast

☆ GenBank *gnn* Accession No. AY566221.
* Corresponding author. Fax: +55 16 33227932.
E-mail address: mcbertol@iq.unesp.br (M.C. Bertolini).

0003-9861/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2004.12.009

proteins. Rabbit skeletal muscle glycogenin [5], the best studied protein, has residue Tyr194 modified by self-glucosylation reaction [6]. In *Saccharomyces cerevisiae*, two genes (*GLG1* and *GLG2*) encoding glycogenin-like proteins have been identified, the proteins showing 55% identity to each other and 33% identity to rabbit muscle glycogenin [7]. Disruption of either gene caused no defect in glycogen accumulation and deletion of both genes is necessary to abolish glycogen storage. Unlike mammalian glycogenin, Glg proteins have multiple Tyr residues for self-glucosylation [8,9]. Characterization of glycogenin-like proteins from other organisms can add new understanding of this enzyme. In *Neurospora crassa*, the existence of a glycoprotein involved in the initiation of glycogen synthesis was initially reported by Takahara and Matsuda [10], and later, Goldraij and Curtino [11] described the purification of a protein with self-glucosylating activity.

The catalytic mechanism of glycogenin is well studied. It is an unusual enzyme since it catalyzes its own modification. The reaction involves the attachment of the first glucose, from UDP-glucose, to a Tyr residue(s) through a glucose-1-*O*-tyrosine linkage and extension to an oligosaccharide chain of up to ~8 glucose residues, through α-1,4-glycosidic linkages [12], which serves as a substrate for glycogen synthase. Glycogenin is a dimeric protein [13,14] and a model was proposed for its enzymatic catalysis whereby modification of one subunit is carried out by the other subunit, in an inter-subunit mechanism [15]. The three-dimensional structure of the rabbit skeletal muscle glycogenin has provided insight into understanding the catalytic action of this complex enzyme [16]. The model confirmed the dimeric nature of the protein but the mechanism of attachment of the first glucose residue is not yet fully understood. From the structure, it was suggested that transfer of the first residue is via an intermolecular reaction whereas the transfer of subsequent residues may be achieved by an intramolecular reaction. Besides its self-glucosylating activity, glycogenin has been shown to be able to *trans*-glucosylate exogenous acceptors, such maltose or maltose derivatives [17].

Little is known about the mechanisms that control glycogenin expression and activity. Control by phosphorylation was proposed by Lomako and Whelan [18] but this assumption has been controversial. Glycogen phosphorylase may control the glucosylation level of glycogenin, modulating its ability to serve as substrate for glycogen synthase [19]. More recently, in a two-hybrid screen, a new protein, called GNIP,[1] was found to interact with rabbit skeletal muscle glycogenin and to activate its self-glucosylation [20,21]. The function of GNIP remains obscure and, to date, no GNIP ortholog has been identified in microorganisms.

In this study, we describe the isolation of a cDNA derived from the *gnn* gene that encodes the *N. crassa* glycogenin, GNN. From in vivo and in vitro studies, GNN has all properties of the better studied glycogenins from mammals and *S. cerevisiae*. Inactivation of the gene completely abolished the glycogen accumulation in *N. crassa*, confirming its role in glycogen biosynthesis and indicating the existence of no other self-glucosylating initiator protein in this organism. In *N. crassa*, GNN regulation is linked to the nutritional status of the organism.

Materials and methods

Strains and growth conditions

Neurospora crassa strains FGSC 424 (WT), Bat9-5a (*a nic*$^-$ *cot-1*$^-$), and FGSC 3957 (*A aro-9 qa-2*$^-$) were purchased from the Fungal Genetics Stock Center (FGSC-University of Kansas, Missouri, Kansas City, USA). The strains were maintained by weekly transfer on slants of Vogel's minimum medium (VM medium) [22] supplemented with 2% sucrose, 10 μg ml^{-1} nicotinic acid (strain Bat9-5a), and 0.15% aromatic amino acids (phenylalanine, tyrosine, tryptophan, and *p*-aminobenzoic acid) (strain FGSC 3957). Liquid cultures were inoculated with 10-day-old conidia (1×10^7/ml final concentration) in minimum Vogel's liquid medium supplemented with 2% sucrose and incubated at 30 °C at 250 rpm. At different times, aliquots (10 ml) were withdrawn, filtered, frozen in dry ice, and stored at −80 °C for further processing. For carbon starvation experiments, liquid cultures were grown for 12 h, at 30 °C and 250 rpm. One aliquot was taken and the remaining culture was filtered. The mycelial pad was suspended in fresh Vogel's minimal medium in which the sucrose concentration was reduced to 0.1%. Aliquots were withdrawn at different times and after that sucrose was added to 2% final concentration and new samples were taken.

Saccharomyces cerevisiae EG328-1A (*Matα trp1 leu2 ura3-52*) and CC9 (*Matα trp1 leu2 ura3-52 glg1::LEU2 glg2::URA3*) were maintained on YPD plates. Yeast transformation followed the lithium acetate method [23], and the transformants were grown in selective medium (SD) lacking tryptophan. *Escherichia coli* strain DH5α was used for plasmid propagation and was grown in Luria–Bertani (LB) medium supplemented with 100 μg ml^{-1} ampicillin. Strain XL-1 Blue was used for library screening experiments and strain BNN 132 (λ Kc lysogenic) was used for plasmid excision from λ vector.

[1] *Abbreviations used:* DTT, dithiothreitol; GNIP, glycogenin-interacting protein; GS, glycogen synthase; GST, glutathione-*S*-transferase; MW, molecular weight; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TLCK, *N*-*p*-tosyl-L-lysine choromethyl ketone; UDPG, uridine diphosphoglucose; VM, Vogel's minimum medium; WT, wild type.