

# Biochemical characterization of *Neurospora crassa* glycogenin (GNN), the self-glucosylating initiator of glycogen synthesis

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**Abstract** Glycogenin acts in the initiation step of glycogen biosynthesis by catalyzing a self-glucosylation reaction. In a previous work [de Paula et al., Arch. Biochem. Biophys. 435 (2005) 112–124], we described the isolation of the cDNA *gmn*, which encodes the protein glycogenin (GNN) in *Neurospora crassa*. This work presents a set of biochemical and functional studies confirming the GNN role in glycogen biosynthesis. Kinetic experiments showed a very low GNN  $K_m$  (4.41  $\mu$ M) for the substrate UDP-glucose. Recombinant GNN was produced in *Escherichia coli* and analysis by mass spectroscopy identified a peptide containing an oligosaccharide chain attached to Tyr196 residue. Site-directed mutagenesis and functional complementation of a *Saccharomyces cerevisiae* mutant strain confirmed the participation of this residue in the GNN self-glucosylation and indicated the Tyr198 residue as an additional, although less active, glucosylation site. The physical interaction between GNN and glycogen synthase (GSN) was analyzed by the two-hybrid assay. While the entire GSN was required for full interaction, the C-terminus in GNN was more important. Furthermore, mutation in the GNN glucosylation sites did not impair the interaction with GSN. © 2005 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

**Keywords:** Glycogen; Glycogenin; Site-directed mutagenesis; Mass spectrometry; Yeast complementation; *Neurospora crassa*

## 1. Introduction

Glycogen, a polymer of glucose, is one of the most important storage carbohydrates in a wide range of species. Its biosynthesis involves three enzymes: glycogen synthase (EC

2.4.1.11), branching enzyme (EC 2.4.1.18), and glycogenin (EC 2.4.1.186), a self-glucosylating protein, which acts in the first step of glycogen biogenesis [1]. Glycogenin has the ability to self-glucosylate, via a 1-*O*-tyrosyl linkage [2], and to polymerize an oligosaccharide up to 10 residues [3]. Once glucosylated, glycogenin acts as the substrate for elongation by glycogen synthase [4], which catalyses the formation of  $\alpha$ -1,4-glycosidic bonds. Both enzymes require the sugar-nucleotide UDP-glucose as substrate. The bulk synthesis of glycogen is completed by the action of the branching enzyme, which introduces branch points, through the formation of  $\alpha$ -1,6-glycosidic bonds [5].

The mechanisms that control glycogen synthesis have been extensively studied and most knowledge has come from studies of mammals and the yeast *Saccharomyces cerevisiae*. Rabbit skeletal muscle glycogenin is self-glucosylated at residue Tyr194, and mutation of this residue led to a protein unable to attach glucoses to itself [6]. In the yeast *S. cerevisiae*, there are two genes, *GLG1* and *GLG2* encoding glycogenin like-proteins Glg1p and Glg2p [7]. In contrast to mammalian glycogenin, Glg proteins possess multiple tyrosine residues involved in self-glucosylation and mutation in all are necessary to inactivate the self-glucosylation, and therefore, for complete abolition of glycogen accumulation [8].

The three-dimensional structure for rabbit skeletal muscle glycogenin, recently solved to 1.9 Å, has provided insights into the catalytic mechanism of this enzyme [9]. The overall fold is very similar to structures of the glycosyltransferase family proteins to which glycogenin belongs. Glycogenin behaves as a dimer in solution [10], and can exist as a pentamer of dimers in the crystals, although the biological relevance of the decamer is uncertain. The dimeric form may be important for function since some mutational studies support a model in which one subunit of a dimer transfers glucose to its partner in an inter-subunit reaction [11]. However, the structure proposed by Gibbons et al. [9] does not fully explain the mechanistic properties of the reaction. Besides its ability to self-glucosylate, glycogenin can also transfer glucose residues to small molecule acceptors, in a trans-glucosylation reaction [12] although there is no evidence to support the physiological relevance of this activity. Both self- and trans-glucosylation activities are strongly enhanced by the presence of  $Mn^{2+}$  ions [4].

In previous work, we described the isolation and characterization of a cDNA derived from the *gmn* gene, which encodes a

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**Abbreviations:** DTT, dithiothreitol; GAPD, glyceraldehyde-3-phosphate dehydrogenase; GNIP, glycogenin-interacting protein; GS, glycogen synthase; GST, glutathione-S-transferase; HEPES, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid); MW, molecular weight; 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 chromomethyl ketone; UDPG, uridine diphosphoglucose; VM, Vogel minimum; WT, wild type

glycogenin-like protein, GNN, in the filamentous fungus *Neurospora crassa* [13]. Expression of GNN in *Escherichia coli* cells resulted in a protein that was very susceptible to proteolysis leading to lower MW species that retained most glycogenin-related activities, such as self- and trans-glycosylation activities and the ability to act as substrate for glycogen synthase. Forms of recombinant GNN, truncated at their C-termini, were more stable against proteolysis and were still active. We also demonstrated that the full-length and truncated proteins could restore glycogen storage to a *S. cerevisiae* mutant strain lacking both *GLG1* and *GLG2* genes.

In the present work, we have extended the functional characterization of the GNN protein by identifying Tyr196 and Tyr198 as the glucosylation sites and determining the contribution of each residue to the self-glycosylation activity. We also analyzed the physical interactions between GNN and *N. crassa* glycogen synthase (GSN) [14] by the yeast two-hybrid assay, to identify the regions in glycogenin important for interaction with glycogen synthase. Our results demonstrate that the C-terminus of GNN is responsible for the interaction with glycogen synthase, although the same region was not important for functional activities, as previously demonstrated [13]. Furthermore, the glucosylation status of GNN seems not to interfere with the interaction with glycogen synthase.

## 2. Materials and methods

### 2.1. Yeast strains, plasmid constructions and site-directed mutagenesis

*Saccharomyces cerevisiae* strains EG328-1A (*MAT $\alpha$  trp1 leu2 ura3-52*) and CC9 (*MAT $\alpha$  trp1 leu2 ura3-52 glg1-2::LEU2 glg2::URA3*) were used in the complementation assays, and strain PJ69-4A [15] was used in the two-hybrid assay. Plasmid pRS424-GPD [16] was used for expression of the Glg1 and Glg2 proteins [7], and the GNN $\Delta$ 306, GNN $\Delta$ 306Y196F and GNN $\Delta$ 306Y196/198F proteins [13] in yeast cells. The plasmids were transformed into yeast cells by lithium acetate method [17] and clones were selected by growth on synthetic medium lacking tryptophan (SD-Trp). The plasmids pGBT9 and pGAD424 [18] were used for expression of the full-length and truncated forms of GSN and GNN, respectively, in the yeast two-hybrid assay. Plasmid pGEX-4T1 (Amersham Biosciences) was used for production of GNN $\Delta$ 360 and GNN $\Delta$ 306 proteins in *E. coli*.

Two-step PCR [19] was used to mutate Tyr196 and Tyr198 to Phe in GNN $\Delta$ 360 and GNN $\Delta$ 306. The plasmid pMOS-GNN (Amersham Biosciences) containing the whole cDNA sequence encoding GNN was used in the first PCR reaction. To make the Tyr196Phe mutation, two DNA fragments were initially amplified by the primers T7 promoter (5'-TAA-TACGACTCACTATAGGG-3')/GNNY196F-R (5'-GGCCGGGATGTACTGGAAGTGGGCGGAGGGCGGT-3') and GNNY196F-F (5'-ACGCCCTCCGCCCACTTCCAGTACATCCCGGCC-3')/U18 (5'-GTTTTCCAGTCACGACG-3'). For the double mutation, Tyr196/198Phe, the two fragments were amplified by the primers GNNY196/198F-F (5'-CTCCGCCCACTTCCAGTTCATCCCGG-3')/U18 and GNNY196/198F-R (5'-CCGGGATGAACTGGAAGTGGGCGGAG-3')/T7. The mutant nucleotides are underlined. The second PCR used the primers GNN-EcoF (5'-GAATT CATGGCGATCA CCAAAGGA-3') and GNN $\Delta$ 3-R (5'-TGGTGTA GGTGCTATGGG-3'), and the amplified products were digested with *ClaI* and *SacII*. The resulting 400 bp fragments were ligated into pGEX-GNN $\Delta$ 360 and pGEX-GNN $\Delta$ 306, previously digested with *ClaI* and *SacII*, generating pGEX-GNN $\Delta$ 360Y196F, pGEX-GNN $\Delta$  360Y196/198F, pGEX-GNN $\Delta$ 306Y196F and pGEX-GNN $\Delta$ 306Y196/198F plasmids.

### 2.2. Production, purification and analysis of mutant GNN proteins

The GNN proteins were expressed in *E. coli* BL21(DE3) as GST-fusion proteins in 500 ml LB medium. Cells were grown at 37 °C to an OD ~ 0.7 and induced by addition of 0.1 mM final concentration IPTG for 3 h at 30 °C. Cells were collected by centrifugation and resuspended in homogenizing buffer (1 × PBS, 1 mM PMSF, 1 mM ben-

zamidine, 5 mM DTT, 0.1 mM TLCK, 1  $\mu$ g/ml pepstatin and aprotinin). Cells were broken by two passages through a French Pressure cell at 18 000 psi. The supernatant was clarified by centrifugation at 18 000 rpm for 20 min and subjected to affinity chromatography on a GStrap column (1 ml) in an Äkta Prime Protein Purification System (Amersham Biosciences). The recombinant proteins were eluted with 10 mM reduced glutathione and dialyzed against 1 l of homogenizing buffer. The self-glycosylation reaction was carried out using 0.8  $\mu$ g of protein in 50 mM HEPES, pH 7.6, 5 mM MnSO<sub>4</sub>, 2 mM DTT, and 19  $\mu$ M UDP-[U<sup>14</sup>C]glucose (263 mCi/mmol) at 30 °C. An aliquot (3  $\mu$ l) was removed from the reaction, spotted onto 2 × 2 cm square P81 chromatography paper and submitted to three washes (30 min each) in 0.5% phosphoric acid and once in ethanol. The papers were dried and counted in a liquid scintillation counter. The remaining reaction was stopped by addition of 4× SDS-PAGE sample buffer, boiled and subjected to SDS-PAGE. The gel was stained, destained, rinsed in water and then in Fluoro-Hance. The gel was dried and autoradiographed.

The proteins (GNN $\Delta$ 306, GNN $\Delta$ 306Y196F, and GNN $\Delta$ 306Y196/198F) were also analyzed by their ability to complement the *glg1* and *glg2* mutations in *S. cerevisiae* strain CC9. Expression of either *GLG1* or *GLG2* genes was used as a positive control and complementation was followed by analysis of glycogen content. Qualitative glycogen measurement was performed by spotting 5  $\mu$ l of a stationary phase culture on SD-Trp plates. Patches were grown for 2 days at 30 °C and then inverted over iodine crystals for detection of glycogen accumulation. Quantitative glycogen measurement was performed as described by Hardy and Roach [20].

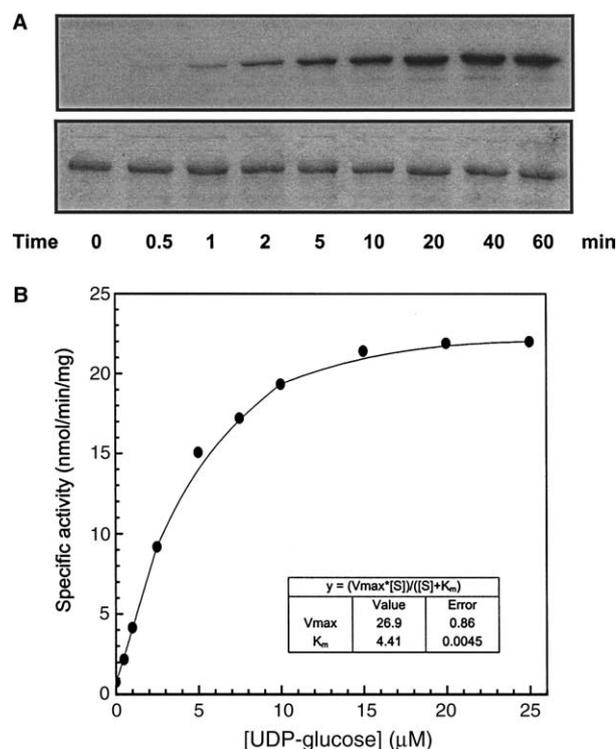


Fig. 1. Kinetics of GNN $\Delta$ 306. (A) Time course of glycogenin self-glycosylation reaction. Purified GST-GNN $\Delta$ 306 (0.8  $\mu$ g) was incubated with UDP-[<sup>14</sup>C]glucose at 30 °C, as described in Section 2. At the indicated times, aliquots were withdrawn and analyzed by SDS-PAGE gel stained with Coomassie blue (lower panel) followed by autoradiography (upper panel). (B)  $K_m$  determination of glycogenin for the substrate UDP-glucose. GST-GNN $\Delta$ 306 (0.8  $\mu$ g) was allowed to self-glycosylate in the presence of the indicated amounts of UDP-[<sup>14</sup>C]glucose at 30 °C for 20 min. After that, aliquots were removed and spotted on P81 chromatography papers, washed in 0.5% phosphoric acid and quantified by scintillation counting.

### 2.3. Mass spectrometry analysis

Recombinant GNN $\Delta$ 360 protein was produced in *E. coli* fused to GST and purified by affinity chromatography. Purified GST-GNN $\Delta$ 360 was allowed to self-glucosylate [13] and was then subjected to SDS-PAGE. The corresponding band was excised from the gel and subjected to in-gel trypsin digestion. The resulting peptides were recovered from the gel slices and analyzed by mass spectrometry at the Proteomics Core Facility, Indiana University School of Medicine, Indianapolis, IN. Briefly, initial characterization of tryptic peptides was carried out by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), using a Micromass M @ LDI-TOF instrument operating in the positive reflectron mode. Further analysis, and characterization of the glucosylated peptide was achieved using a capillary liquid chromatography system, coupled with a quadruple time of flight (Q-TOF) mass spectrometer (Micromass) fitted with a Z-spray ion source. Separation of the peptides was carried out on a reverse-phase capillary column running at 300 nl/min. The mobile phase consisted of a linear gradient from 100% solution A (0.1% formic acid/3% acetonitrile, v/v, in water) to 70% solution B (0.1% formic acid/97% acetonitrile, v/v, in water) for 50 min followed by a 10-min gradient to 100% solution B. Mass spectra were acquired in positive ion mode.

### 2.4. GSN and GNN plasmid constructions for two-hybrid assays

The entire coding sequence of *gmn* cDNA [13] was amplified and inserted into pGAD424 vector leading to the pGAD424-GNN plasmid. The truncated GNN proteins GNN $\Delta$ 613, GNN $\Delta$ 545, GNN $\Delta$ 360, GNN $\Delta$ 306, and GNN $\Delta$ 245 were prepared by amplifying the DNA fragments with the sense primer GNN-EcoF (sequence above) and antisense primers containing stop codon at internal positions of the cDNA, and then, insertion of the fragments into pGAD424 plasmid. The pGAD424-GNN490-664 and pGAD424-GNN337-664 plasmids were constructed by amplification of the sequences using internal primers in the *gmn* cDNA sequence and the antisense primer NcGN-3R (sequence above). *EcoRI/SacII* DNA cassettes from pGEX-GNN $\Delta$ 360Y196F and pGEX-GNN $\Delta$ 360Y196/198F were exchanged into pGAD424-GNN to generate the pGAD424-GNNY196F and pGAD424-GNNY196/198F plasmids, respectively. *EcoRI/SalI* DNA cassettes from pGEX-GNN $\Delta$ 360Y196F and pGEX-GNN $\Delta$ 360Y196/198F were exchanged into pGAD424-GNN $\Delta$ 306 to construct the pGAD424-GNN $\Delta$ 306Y196F and pGAD424-GNN $\Delta$ 306Y196/198F

plasmids, respectively. The pGBT9-GSN plasmid resulted from insertion of the entire ORF of the *gsn* cDNA, amplified with the oligonucleotides GSNth-F (5'-GAATTCATGGCCCCACGACAACCGTG-3')/GSNth-R (5'-CTGCAGTTACCTGGTGCCGTTG-3') [14], into pGBT9 vector. cDNAs encoding the truncated GSN proteins GSN $\Delta$ 387 and GSN $\Delta$ 624 were constructed by PCR amplification using the oligonucleotide GSNth-F and antisense oligonucleotides containing stop codon, and inserting the fragments into pGBT9 plasmid. Sense oligonucleotides hybridizing at internal sequences of the *gsn* cDNA and the antisense oligonucleotide GSNth-R were used to amplify DNA fragments encoding the proteins GSN150-706 and GSN347-706, and insertion of the DNA fragments into pGBT9 vector.

### 2.5. Yeast two-hybrid assay

To analyze the interactions between GSN and GNN proteins, pGBT9-GSN plasmid was co-transformed with the different pGAD424-GNN constructs in the yeast strain PJ69-4A. In the same way, pGAD424-GNN plasmid was co-transformed with the various pGBT9-GSN constructs. The transformants were selected by plating on the selective medium SC-Trp-Leu and after on SC-Trp-Leu-His + 5 mM 3 aminotriazole (3-AT) plates (2% glucose, 0.67% yeast nitrogen base without amino acids and 0.15% amino acid mixture). The presence of all proteins was confirmed by Western blot analysis using either anti-GSN or anti-GNN antibodies. Quantitative analysis was performed by liquid  $\beta$ -galactosidase assay [21] from cells collected after 24 h of growth in liquid cultures.

## 3. Results and discussion

### 3.1. Kinetics of GNN

In previous work, we described the isolation of the *N. crassa gmn* cDNA and demonstrated that the protein (GNN) had several properties associated with glycogenins [13]. The recombinant GNN was very susceptible to proteolysis toward the C-terminus, resulting in smaller polypeptides that nonetheless retained most enzymatic activity. Since GNN is one of the largest glycogenin-like proteins isolated so far, we

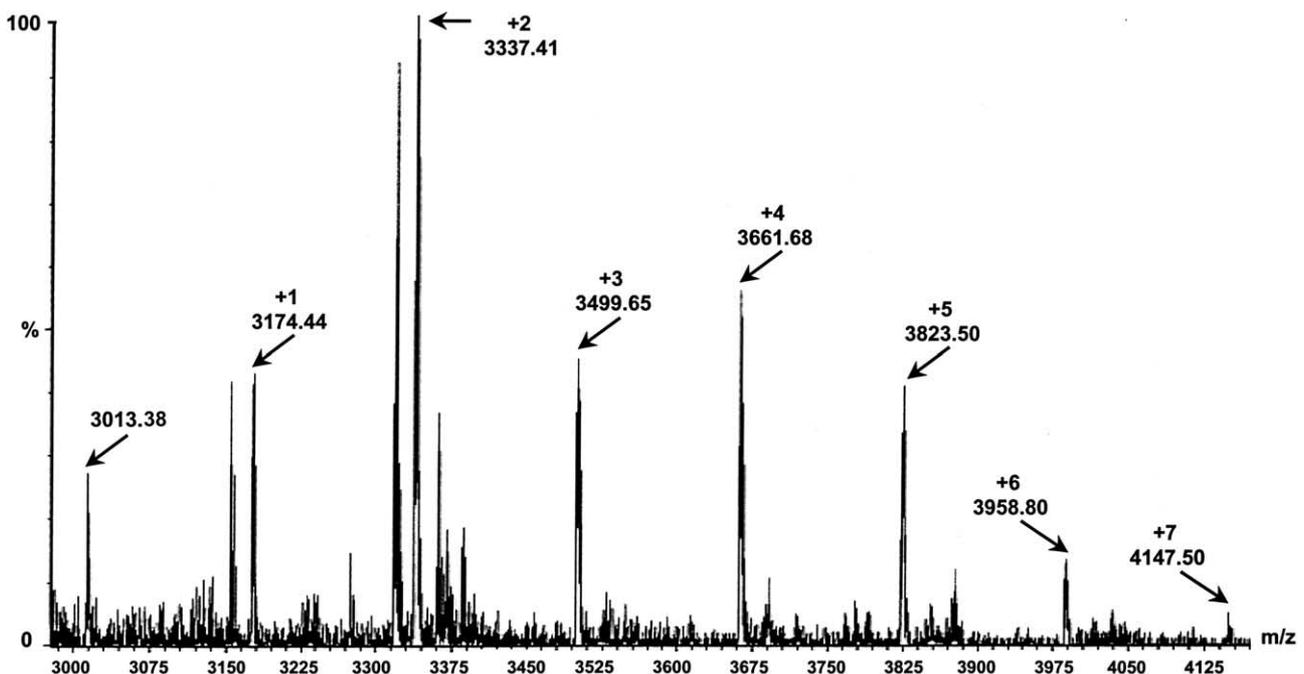


Fig. 2. Analysis of glycogenin glucosylation by mass spectrometry. The peptides generated by trypsin digestion of glucosylated GST-GNN $\Delta$ 360 were analyzed by MALDI-TOF. The ion spectrum is shown in the mass range 3000–4000. The peak masses are shown along the spectrum and refer to the additional glucose units added to the unmodified peptide (mass 3013.38).

questioned whether the long C-terminal region was important for the glycogenin activities. To investigate this hypothesis, we extended the biochemical analysis of GNN. Initially, we determined the kinetic properties of GNN by using a truncated protein, which lacks 358 amino acid residues from the C-terminal region (GNN $\Delta$ 306). We elected to use the truncated protein because expression of the full-length protein results in a heterogeneous mixture due to C-terminal proteolysis [13]. This protein was produced in *E. coli* as a GST-tagged protein. Fig. 1A shows the time course of the self-glucosylation reaction when the recombinant protein was incubated in the presence of UDP-[U- $^{14}$ C]glucose and subsequently analyzed by SDS-PAGE. The reaction progressed linearly up to 20 min and then reached a plateau at which no further incorporation occurred. A slight reduction in mobility was observed as a function of the incorporation of glucose residues. The recombinant protein self-glucosylated with a  $K_m$  toward the substrate UDP-glucose of about 4.41  $\mu$ M (Fig. 1B). The kinetic data on the self-glucosylation activity of GNN are in agreement with data described for mammalian [6,22,12] and yeast [7] glycogenins. The  $K_m$  value is low, similar to the rabbit protein (4.5  $\mu$ M), and the self-glucosylation reaction is activated by  $Mn^{2+}$  ions.

### 3.2. Analysis of GNN self-glucosylation site

To identify the acceptor site of glucose in the self-glucosylation reaction, we used the GNN $\Delta$ 360 protein fused to GST. The full-length protein is so susceptible to proteolysis when produced in *E. coli* that it is impossible to produce more than a complex mixture of proteolytically related species. Therefore, we used a truncated form of GNN (GNN $\Delta$ 360), which lacks 304 amino acids from the C-terminal region and which we know to be catalytically active [13]. This form still undergoes some proteolysis to generate another species but this did not interfere with the analysis. The GST-GNN $\Delta$ 360 was allowed to self-glucosylate and the band corresponding to the non-proteolysed protein was excised from the SDS-PAGE gel, and analyzed by mass spectroscopy after trypsin digestion. A peptide corresponding to amino acid residues 179–203 (NTYNRLSFTYNVTPSAHYQYIPAYK) was identified in GNN to be modified by addition of glucose residues. This peptide still contains an arginine residue (Arg183) that is, most likely, a missed site for trypsin cleavage. This peptide contains the Tyr196 residue that aligns with Tyr194 and Tyr232, which were demonstrated to be the glucosylation sites in the rabbit skeletal muscle glycogenin [6], and in the yeast Glg2p [7] proteins, respectively. The mass spectrum (Fig. 2) indicated the presence of a peak corresponding to the parent peptide (3013.38 atomic mass observed) and seven additional ions of higher mass. The peaks represented a successive increase of 162 mass units, which corresponds to the mass difference of one additional glucose residue. The signal corresponding to +7 glucoses is considerably weaker and the most prominent peak corresponded to the parent peptide plus two glucose residues. Sequencing of the peptide showed that the Tyr196 residue was the primary modified residue (not shown).

### 3.3. Analysis of mutant GNN proteins

Site-directed mutagenesis was used to generate GST-GNN $\Delta$ 360 and GST-GNN $\Delta$ 306 proteins with Tyr196 mutated to Phe. Assays of self-glucosylation of the two mutant forms

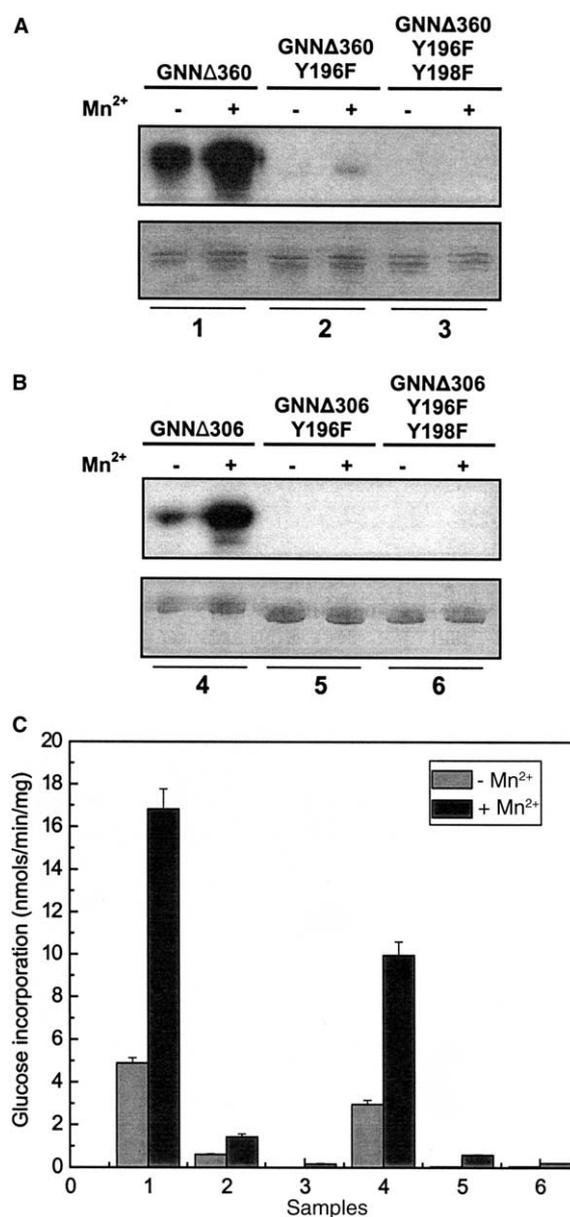


Fig. 3. Self-glucosylation of WT and mutant glycogenins. WT proteins (GNN $\Delta$ 360 and GNN $\Delta$ 306) and mutant proteins (GNN $\Delta$ 360Y196F, GNN $\Delta$ 360Y196/198F, GNN $\Delta$ 306Y196F and GNN $\Delta$ 306Y196/198F) were analysed by the ability to self-glucosylate. The proteins (0.8  $\mu$ g) were incubated with UDP-[ $^{14}$ C]glucose at 30  $^{\circ}$ C for 20 min. Aliquots were removed and spotted in P81 papers and counted by liquid scintillation (C). The remaining reaction was stopped by adding sample buffer (4X) and boiling. Samples were analyzed by SDS-PAGE gel stained with Coomassie blue (lower panels), followed by autoradiography (upper panels). (A) Self-glucosylation of GNN $\Delta$ 360 (1), GNN $\Delta$ 360Y196F (2) and GNN $\Delta$ 360Y196/198F (3) proteins, (B) Self-glucosylation of GNN $\Delta$ 306 (4), GNN $\Delta$ 306Y196F (5) and GNN $\Delta$ 306Y196/198F (6) proteins.

revealed a residual incorporation of  $^{14}$ C-radiolabelled glucose in the GST-GNN $\Delta$ 360Y196F protein (Fig. 3A, lane 2) not present in the GST-GNN $\Delta$ 306Y196F protein (Fig. 3B, lane 5). This result suggested the existence of another residue that could be a secondary glucosylation site in GNN. Thus, the double mutant GST-GNN $\Delta$ 360Y196/198F and GST-GNN $\Delta$ 306Y196/198F proteins were generated by site-directed

mutagenesis and produced in *E. coli*. The radiolabelling observed in the GST-GNNA360Y196F mutant was abolished in the double mutant GST-GNNA360Y196/198F (Fig. 3A, lane 3). Consistent with this result, no incorporation was seen in the double mutant GST-GNNA360Y196/198F (Fig. 3B, lane 6). Quantitative analysis of incorporation revealed that the rate of self-glucosylation of the GST-GNNA360 and GST-GNNA306 proteins were approximately 16.81 and 9.95 nmol/min/mg, respectively, while in the mutant GST-GNNA360Y196F this value was approximately 1.42 nmol/min/mg (Fig. 3C). Double mutants containing Tyr196Phe and Tyr202Phe mutations were also generated but the results indicated no glucosylation at Tyr202 (data not shown). In summary, the data suggest that GNN contains two glucosylation sites, Tyr196 and Tyr198. The yeast Glg2p isoform has two glucosylation sites (Tyr230 and Tyr232), which are very close together, but in this protein both sites contributed equally to the self-glucosylation [7]. A recent study [23] has shown that in bacterially produced Glg2p, both Tyr residues can be modified simultaneously in the same Glg2p monomer.

The relevance of two glucosylation sites was also analyzed in vivo, by expression of the mutant forms of GNNA306 in a yeast strain (CC9) lacking both glycogenin genes (*glg1 glg2*). Fig. 4 shows the glycogen accumulation in cells complemented

with GNNA306 and the mutant proteins GNNA306Y196F and GNNA306Y196/198F (Fig. 4A). Expression of the truncated protein GNNA306 was able to restore the glycogen levels close to the physiological levels when compared with both the WT cells or CC9 cells expressing either *GLG1* or *GLG2* genes. Expression of the single mutant GNNA306Y196F correlated with a reduced but substantial glycogen accumulation, corresponding to 30% of the glycogen content observed in the cells complemented with the WT GNN (Fig. 4B, sample 6). Moreover, glycogen was not accumulated when the double mutant protein GNNA306Y196/198F was expressed in the mutant yeast cells (Fig. 4B, sample 7). The contribution of the Tyr198 residue in the self-glucosylation reaction was more evident when in vivo analysis was performed. This result confirms both Tyr196 and Tyr198 residues in GNN protein as the physiological relevant glucosylation sites, although Tyr196 is the most important site.

### 3.4. Physical interactions between glycogenin and glycogen synthase

We undertook a systematic truncation mutagenesis protocol to map the regions that might be important for physical interaction between glycogenin (GNN) and glycogen synthase (GSN). Little information concerning this issue is available from studies on yeast proteins [7]. For this purpose, we generated deletions in both proteins and used the yeast two-hybrid assay to evaluate the possible domains involved in the GNN–GSN interaction. Fig. 5A shows a schematic representation of the truncated forms that were assayed. Western blot with anti-GSN and anti-GNN antibodies confirmed the presence of proteins. In one set of assays, GSN protein was used as bait with a series of truncated GNN proteins as prey, and the interactions were evaluated by quantifying the  $\beta$ -galactosidase activity (Fig. 5B). Entire GNN and GSN proteins interacted strongly to each other, as expected. Deletion of the 51 (GNNA613) and 119 (GNNA545) C-terminal amino acids reduced the interaction between the two proteins to about 82% and 69%, respectively. Moreover, the truncated mutants GNNA360, GNNA306, and GNNA245 interacted much more weakly with GSN, yielding only about 10% of the reporter activity detected with wild type GNN. On the other hand, a polypeptide comprising only the C-terminus of GNN (GNN490–664), interacted poorly with GSN and this was only slightly enhanced by enlarging it to span residues 337–664 (GNN337–664). Taken together, these results argue that the C-terminal region of GNN, starting at residue 360, dominates the interaction with GSN but that interaction depends on the integrity of the N-terminus. Finally, the interaction between the two proteins was barely affected by mutations in one or both glucosylation sites, demonstrating that the glucosylation status of glycogenin must not be important in the interaction with GSN. Cheng et al. [7] have proposed that the extreme C-terminus domain in Glg2p was required for interaction with Gsy2p since this fragment was isolated in a two-hybrid screening using Gsy2p as bait. The C-terminal domain in GNN shares very low homology to Glg2p and may function somewhat differently.

In another set of experiments, GNN was used as bait and four deletions in GSN were assayed. The N- and C-terminal regions of GSN (GSNA387 and GSN347–706) were individually assayed and neither allowed significant interaction with GNN. Extension of the C-terminus in GSN150–706 did not

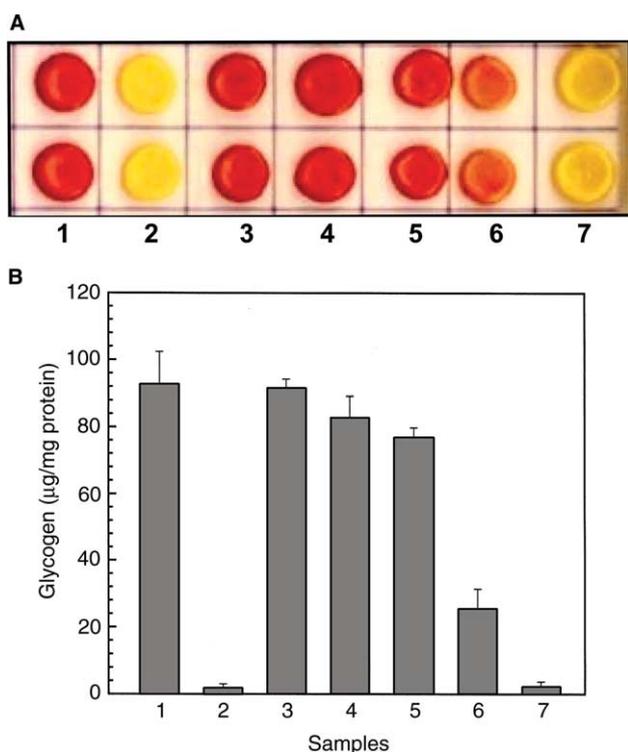


Fig. 4. Functional complementation of *S. cerevisiae* glycogenin mutant strain by *N. crassa* glycogenin. GNNA306 and mutant proteins GNNA306Y196F and GNNA306Y196/198F were expressed in *glg1 glg2* yeast cells (CC9) from high copy number vector pRS424 under the control of the GAPD promoter. Samples 1 and 2, WT and CC9 strains, respectively, harboring pRS424; samples 3 and 4, CC9 cells overexpressing Glg1p and Glg2p, respectively; samples 5, 6, and 7, CC9 cells overexpressing GNNA306, GNNA306Y196F and GNNA306Y196/198F, respectively. (A) Iodine staining of colonies grown on SD-Trp plate for 2 days at 30 °C. (B) Glycogen content. Glycogen was determined enzymatically as described in Section 2.



due the ability of glycogen synthase to interact with the oligosaccharide chain of glycogenin. Even though the interactions take place in the nucleus in the two-hybrid analysis GNN must be translated in the cytosol, and will likely be glucosylated and translocated to the nucleus carrying an oligosaccharide chain attached. In fact, the extension of the oligosaccharide chain has been proposed to play a role in the ability of glycogen synthase to elongate self-glucosylated glycogenin in mammals [24]. However, our results using single or double mutant proteins argue that GNN–GSN interaction does not depend on the glucosylation status of glycogenin. In this case, other protein–protein determinants may explain the capacity of those two proteins to interact, albeit not strongly. Recent reports describing the existence of a protein called GNIP [25], which interacts with mammalian glycogenin, suggest that multimeric complexes may involve glycogenin and glycogen synthase [26]. Although no GNIP counterpart has been identified in microorganisms, we cannot exclude the possibility that other proteins may exist, and to be implicated on the physical interactions and activity of both glycogenin and glycogen synthase.

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