Glycosylated DNA-Binding Proteins from Filamentous Fungus, Aspergillus oryzae: Modification with N-Acetylglucosamine Monosaccharide through an O-Glycosidic Linkage

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The existence of glycosylated DNA-binding proteins was demonstrated in a whole cell extract from a filamentous fungus, Aspergillus oryzae. The proteins were specifically eluted from a DNA-cellulose column by the eluate containing shared double-stranded DNA and were detected by wheat germ agglutinin (WGA)-probing. The apparent molecular masses of these proteins on SDS–PAGE were 140 kDa, 115 kDa, 105 kDa, 68 kDa, and 60 kDa. The labeling of the proteins by uridine 5'-diphosphate(UDP)-[14C]galactose using galactosyltransferase showed the same electrophoretic pattern with the WGA-probing. The [14C]galactose-labeled saccharides were released from the proteins by mild-base treatment but not by N-glycopeptidase F digestion, indicating the O-glycosidic linkage of the saccharide chain attachment to proteins. The [14C]galactose-labeled saccharides co-migrated with galactose-(β1→4)-N-acetylglucosaminitol on a silica gel plate. Thus, it was seen that several proteins which had the DNA-binding activity were modified by N-acetylglucosamine monosaccharide through an O-glycosidic linkage in A. oryzae.

In recent years, a wide distribution of novel glycosylated-proteins modified by N-acetylglucosamine monosaccharide through O-glycosidic linkages (O-GlcNAc) have been demonstrated in various eukaryotic organisms.1–3 Unlike other glycoproteins, the O-GlcNAc-bearing proteins have been found to be localized in the nuclear and cytoplasmic compartments of the cell.4,5 Among many O-GlcNAc-bearing proteins, it has been reported that the several DNA-binding proteins involved in the RNA-polymerase II-mediated transcription are also glycosylated in human, calf, mouse, and Drosophila cells.6–10

Although it is still unclear that the unique O-GlcNAc modification is biologically important, several examples of the changes of O-GlcNAc density on nuclear proteins11–13 and the findings of specific O-GlcNAc transferases14 and glucosidases,15 suggest that the unique O-GlcNAc modification is a highly dynamic post-translational modification quite similar to protein phosphorylation. Despite the notable findings about the O-GlcNAc modification and the isolation of numerous numbers of nuclear proteins including DNA-binding proteins from yeast,16 no clear evidence has been demonstrated that the glycosylated DNA-binding proteins exist in lower eukaryotes. Two groups reported independently that reproducible signals were not detected by WGA-probing of the protein blots of yeast nuclei17,18 suggesting the lack or, if any, a trace of O-GlcNAc-modified DNA-binding proteins in yeast nuclei.

In this paper, we report the existence of several DNA-binding proteins that are modified by GlcNAc monosaccharide through O-glycosidic linkage in a filamentous fungus, A. oryzae. The results may suggest the existence of a function of O-GlcNAc found on the DNA-binding proteins in A. oryzae similar to that of human transcription factors.

Materials and Methods

Strains, media, and reagents. The A. oryzae strain RIB4019 isolated by Nojiri et al. was grown in DP medium20 for 22 h at 30°C. The reagents for the enhanced chemiluminescence reaction, salmon testes DNA (type III), human milk galactosyltransferase, and recombinant N-glycopeptidase F were purchased from Amersham, Sigma, Boehringer, and Genzyme, respectively. The marker proteins for the apparent molecular masses were purchased from Pharmacia (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and z-lactalbumin) for silverstaining or from Bio-Rad (prestained standards) for the WGA-staining and the galactosylation.

Preparation of whole cell extracts. Mycelia (approximately 12 grams) were washed with chilled water and suspended in homogenization buffer containing 25 mM Hepes-NaOH (pH 7.5), 50 mM KCl, 5 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol, 0.3 mM ammonium sulfate, 1 mM phenylmethylsulfonyl fluoride (PhMeSO4F), 2 μg/ml chymostatin, 10% glycerol, and the proteinase inhibitors mixture (PI-mix; 5 μg/ml antipain, 2 μg/ml apronitin, 50 μg/ml leupeptin, and 1 μg/ml pepstatin A). The mycelia suspension was homogenized by 2 × 30 s by a Brown homogenizer in the presence of 0.45 × 0.50 mm glass beads. After debris were removed by centrifugation for 10 min at 15,000 × g, the proteins were concentrated by ammonium sulfate at 80% saturation and dialyzed against the homogenization buffer in which ammonium sulfate was omitted and 0.1% Tween 20% aprotinin and 10 μg/ml chymostatin were used instead of 2% Tween and 2% aprotinin, respectively. The resulting whole cell extracts containing 20–30 μg/ml proteins were stored at −80°C until use. All the procedures were done at 4°C, on ice or in the cold gas flow of carbon dioxide.

DNA-Affinity chromatography. One milliliter of the whole cell extracts (about 20 mg of proteins) was put onto a Sephadex G-25 column to exchange the buffer with buffer D, which consisted of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM EDTA, 0.5 mM dithiothreitol, 0.05 mM PhMeSO4F, 2 μg/ml chymostatin, 10% glycerol and PI-mix. The eluate was centrifuged for 1 h at 100,000 × g to precipitate the undissolved material, and then was put onto the native DNA-cellulose (Pharmacia) column (1 ml, 0.8 × 2 cm). After the extensive wash with buffer D, the adsorbed proteins were eluted by the same buffer containing 1 mM NaCl and pooled. The PI-mix was omitted from the buffers used for washing and elution, thus the proteinase inhibitors involved in the eluent were

Abbreviations: WGA, wheat germ agglutinin; GlcNAc, N-acetylglucosamine; PhMeSO4F, phenylmethylsulfonyl fluoride; GlcNAcitol, N-acetylglucosaminitol.
A

![Image](image-url)  
**Fig. 1.** Detection of Glycosylated Proteins in the Fraction from DNA-Cellulose Column.  
(A) The whole-cell extracts from *A. oryzae* were put on a native DNA-cellulose column (1 ml gel volume) and after extensive washing, the column was further washed with one column volume of buffer D containing 6 mM sodium phosphate (lanes 1–7). The proteins were recovered by a solution containing 1 M sodium chloride (lanes 8–13). The proteins for (example, 3.0 µg in lane 8) resolved on SDS-PAGE on the gel were directly silver stained (lanes 14 and 15) or WGA-probed as described in Materials and Methods (lanes 1–13). Lanes 1 and 14, whole cell extracts; lane 15, salt-eluative equivalent to lane 8. (B): Buffer D containing 2 mg/ml shared salmon testes DNA instead of 6 mM sodium phosphate was used to achieve DNA-specific elution from DNA-cellulose column (0.8 ml gel volume) (lanes 3–6). The remaining proteins were successively eluted by the solution containing 1 M NaCl (lanes 7–12). Lane 1, whole cell extracts. Arrowheads (I, II, III, and IV) and a bracket (II) indicate the positions of the bands detected by WGA-probing.

**Results**

**Detection of the glycosylated DNA-binding proteins**

The whole-cell extracts from *A. oryzae* were put onto a native DNA-cellulose column and after extensive washing with 10 ml of buffer D, the column was further washed with one column volume of buffer D containing 6 mM sodium phosphate. Finally, the proteins retained in the column were recovered by a solution containing 1.0 M sodium chloride. The proteins in each fraction were resolved on a SDS-polyacrylamide gel. The proteins were transferred onto the membrane and incubated with WGA-peroxidase conjugate, which recognizes N-acetylglucosamine (GlcNAc) residues. The migration positions of the lectin-bound proteins on the membrane were made visible by the chemiluminescence reaction catalyzed by peroxidase (Fig. 1A, lane 1–13).

As judged from the silver-staining of the gel, a limited number of proteins was recovered from the DNA-cellulose column (Fig. 1A, lane 15 as compared with lane 14). Among the proteins contained in the eluate shown in Fig. 1A, lane 15, several proteins were detected by WGA-staining at the apparent molecular masses of 140 kDa, 115 kDa, 105 kDa, 68 kDa, and 60 kDa. Since the two bands at 115 kDa and 105 kDa were close, the positions of these bands were indicated by bracket designated II, while the other bands were indicated by arrowheads in Fig. 1A. These bands were detected only in the eluate by 1 M sodium chloride but not in the 6 mM phosphate eluate. The bands were WGA-specific, since concanavalin A-peroxidase instead of WGA-peroxidase failed to detect the proteins (data not shown). All the bands detected by the WGA-probing completely disappeared after Pronase E treatment before
SDS–gel electrophoresis, indicating that the bands were derived from protein factors (data not shown).

To exclude the possibility that the proteins of interest may be retained in the native DNA–cellulose resin through a nonspecific interaction, the DNA-specific elution was done by using an eluent containing shared double-stranded DNA instead of 6 mM phosphate (Fig. 1B, lane 3–6). The proteins detected at the positions of I to III in Fig. 1A immediately started to be eluted by the solution containing 2 mg/ml salmon testes DNA, indicating a DNA-specific interaction.

The band IV in Fig. 1A could not be detected clearly in the DNA eluate in Fig. 1B because of a strong smeared background even after the longer exposure. In our experiment, the sample containing shared DNA had a higher background than the sample lacking DNA. We assumed that the interaction between the positive charge of WGA and the negative charge from the phosphogroups of DNA might cause the higher background in WGA-probing.

To confirm the GlcNAc-specific interaction of WGA-peroxidase, 0.1 M GlcNAc or 0.1 M glucose as a “mock” was added in the reaction mixture during the binding reaction of WGA-peroxidase. As shown in Fig. 2A, the addition of GlcNAc completely inhibited the WGA binding to the protein corresponding to bands I–IV (lane 3) while the addition of glucose did not (lane 2). Further, weak acid treatment of the blot, which could remove sialic acid from the saccharide moiety, did not have any significant effects on the bands I–IV by WGA-probing (Fig. 2B, lane 2) while the same treatment almost completely blocked the detection of fetuin a glycoprotein that has sialic acid (Fig. 2B, lane 4). These observations indicate that the proteins of bands I–IV detected by the WGA-probing are modified by a saccharide moiety containing GlcNAc but not sialic acid.

\[^{14}C\text{Galactose labeling by galactosyltransferase}\]

To confirm the GlcNAc-modification of DNA-binding proteins in A. oryzae, the proteins eluted from the DNA-cellulose column were galactosylated by galactosyltransferase, which transfer galactose residues of UDP-[U-\(^{14}\text{C}\)]galactose onto terminal GlcNAc residues of glycoproteins.\(^{18}\)

The autoradiogram of the SDS-polyacrylamide gel displayed five clear bands at the apparent molecular masses of 140 kDa (band I), 115 kDa + 105 kDa (doublet bands II), 68 kDa (band III), and 60 kDa (band IV) (Fig. 3A, lanes 2, 3), indicating the similar migration patterns obtained by WGA-probing. A limited number of the galactosylated proteins were found to be DNA-specific binding proteins, when compared with the result of whole cell extract (Fig. 3A, lane 1). No bands could be detected in the control experiment, where the elution buffer containing the shared DNA was used instead of the protein sample (Fig. 3A, lane 4). The migration positions of the bands I–IV in Fig. 3A were confirmed to correspond exactly to the positions of the bands I–IV in Fig. 1A by superimposition on one gel (data not shown). Therefore, it is indicated that the DNA-binding proteins, corresponding to bands I–IV in Fig. 1A and Fig. 3A, are modified with sugar molecules involving the terminal GlcNAc residue.

The two different detection methods, WGA-probing and \[^{14}C\text{Galactose labeling}\], showed similar characteristics, e.g., the intensity of band I was almost the same with that of the upper bands at position II in the DNA-eluate by both detection methods (Fig. 1B, lane 4 and Fig. 3A, lane 3), while the band I in the salt-eluate showed much higher density than the bands II in the same eluate (Fig. 1A, lane 8 and Fig. 3A, lane 2). On the other hand, the intensity of the band IV detected by \[^{14}C\text{Galactose labeling}\] (Fig. 3A, lanes 2 and 3) was much stronger than that by WGA-probing (Fig. 1A, lane 8 and Fig. 1B, lane 4). Since
the detection of the band IV in the DNA-elute by WGA-probing is not clear, it is not evident whether the protein corresponding to band IV was eluted effectively from the column by DNA solution or a significant amount of the protein was still retained in the column after the DNA-elution, as was retained the protein corresponding to band I. However, the intensity of the band IV in the DNA-elute must be much weaker than in the salt-elute if the band IV protein was not effectively eluted from the column, as observed in band I. Therefore, the band IV protein, which showed almost the same intensity in both the salt-elute and the DNA-elute (Fig. 3A, lanes 2 and 3), was thought to be effectively eluted by the DNA solution.

**Linkage analysis**

To address the glycosidic linkage of GlcNAc residue(s), the [14C]galactose-labeled DNA-binding proteins were denatured by boiling in the presence of 0.15% SDS, and were treated with N-glycopeptidase F, which selectively removes N-linked oligosaccharides from polypeptides. Under these reaction conditions, [14C]galactose-labeled saccharide chains attached to polypeptides through N-glycosidic linkages (ovalbumin as a model) were completely removed as shown in Fig. 3B, lanes 3 and 4. However, the N-glycopeptidase F digestion of [14C]galactose-labeled A. oryzae DNA-binding proteins indicated no changes in the intensity of the bands I–IV after the reaction for 18 h at 37°C (Fig. 3B, lanes 1 and 2).

Further to confirm the O-glycosidic linkage, the total [14C]galactosylated proteins contained in the DNA-eluate from the DNA-cellulose column were treated with mild-base as described in Materials and Methods. The reaction mixture was put onto a Sephadex G-50 column, and the radioactivity in each fraction eluted from the column was measured by a liquid scintillation counter. As shown in Fig. 4, greater than 98% of the [14C]-labeled saccharides derived from the DNA-binding proteins eluted in the inclusion volume of the Sephadex G-50 column after the β-elimination reaction. Without β-elimination, the proteins eluted quantitatively in the exclusion volume of the column. Thus, it is indicated that the major [14C]-labeled DNA-binding proteins including bands I–IV were modified with saccharide(s) through O-glycosidic linkage.

**Identification of the 14C-labeled saccharide**

The 14C-labeled saccharide eluted in the inclusion volume from the Sephadex G-50 column was put onto a Bio-Gel P-2 column as described in Materials and Methods. The fractions were collected and analyzed by liquid scintillation counting. The radioactivity was detected as a single peak only in the fractions between Gal(β1→4)GalNAc and GlcNAc, as shown in Fig. 5A, which was exactly the same elution position of galactose-(β1→4)-N-acetylgalcosaminitol (Gal-(β1→4)GalNAc).5 The fractions containing the radioactivity were collected and analyzed by high performance thin layer chromatography as described in Materials and Methods. Figure 5B shows that all the radioactivity migrated at the position of the Gal(β1→4)GalNAc standard. These results, together with the results described above, clearly indicate that the DNA-binding proteins from A. oryzae detected in this work were modified with GlcNAc monosaccharide through the O-glycosidic linkage.

**Discussion**

Several DNA-binding proteins that are modified by GlcNAc monosaccharide through O-glycosidic linkage were detected in a filamentous fungus, A. oryzae. All the proteins were discharged from the native DNA-cellulose column specifically by the solution containing native DNA. It is not likely that the proteins were retained in the column through simple electrostatic interaction with the negative charge on the surface of the DNA because these proteins were not discharged from the column by a solution containing 6 mM phosphate, which provides a negative charge equivalent to the DNA solution.

Among the proteins detected in this work, roughly half of the total amount of the band I protein, which was retained in the column after the DNA-elution, was further eluted by the successive salt-elution, while the proteins at positions II and III were almost completely discharged from the
column by the DNA-elution. This may suggest the existence of two different proteins of the same migration mobility; one is DNA-specific and the other is non-specific. However, when the DNA-cellulose column that retained the proteins was treated with micrococal nuclease in the presence of CaCl₂, which removed the immobilized DNA from the gel matrix,²⁹ no significant bands were detected in the successive salt-eluante though the treatment without nuclease did not affect the protein-DNA interaction (data not shown). Therefore, it is interpreted that all the fractions of the band I protein were retained in the column through DNA-specific interaction and that a very low dissociation constant (high affinity for DNA) of band I protein may result in ineffective elution by the DNA-solution.

Recently, Jackson and Tjian reported that several factors involved in the RNA-polymerase II-mediated transcription in mammalian cells⁶⁰ were glycosylated by multiple GlcNAc monosaccharide through O-glycosidic linkage, and that the O-linked GlcNAc of the human GC-box binding protein, Sp1, played an important role in the transcriptional activation.⁶¹ However, no clear evidence was found that the O-GlcNAc containing DNA-binding proteins were distributed in lower euchromes, like fungi or yeast. To our best knowledge, this is the first paper to report the existence of O-GlcNAc-modified DNA-binding proteins in filamentous fungi. The fungus reported here instead of yeast may provide a good model system for studying the function of the unique O-GlcNAc-modification on the DNA-binding proteins in lower euchromes.

It was reported that labeling of Sp1 with galactose resulted in an approximately 5-kDa increase in the apparent molecular mass of Sp1 on SDS-PAGE, in consistent with that Sp1 molecule was estimated to contain approximately eight GlcNAc residues.⁶² To estimate the glycosylation mass of A. oryzae DNA-binding proteins, [¹⁴C]-galactose-labeled proteins and unlabeled proteins were run in the same gel, and detected by autoradiogram and WGA-probing, respectively. Each band I–IV corresponding to labeled and unlabeled proteins migrated exactly at the same position (data not shown). Since the resolution of the SDS-PAGE in our experiments was less than 5 kDa, ranging from 60 kDa to 140 kDa, this indicates that the numbers of O-GlcNAc on the proteins corresponding to bands I–IV are fewer than eight. Therefore, the sensitive detection of the proteins of bands I–IV is not due to their heavy glycosylation but due to the abundance of those proteins in the cell.

Under our experimental conditions, less than 100 ng of ovalbumin was reproducibly detected with a strong intensity by WGA-probing (data not shown). Although the sensitivity to detect the glycosylated protein by WGA-probing strictly depends on the characteristics and the number of the glycosylations, the amount of each protein corresponding to bands I and II is roughly calculated to be 1 µg per 1 g of wet cells. Since this is almost the same order of magnitude with the quantity of Sp1,³⁰ the proteins are thought to be ubiquitous DNA-binding proteins, like the proteins related to the chromatin structure or general transcription.

The existence of the O-GlcNAc-modification of the DNA-binding proteins in A. oryzae may suggest that the existence of the same function of the O-GlcNAc-modification in filamentous fungi with that in mammals. Thus, it is interesting to investigate the function of these glycosylated proteins and to analyze the role of O-GlcNAc-modification in A. oryzae. The rough measurement showed that approximately 13,000-fold purification will be necessary to isolate the proteins corresponding to bands I and II, which will enable us to clone the corresponding genes.

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References