Glucomannoproteins in the cell wall of Saccharomyces cerevisiae contain a novel type of carbohydrate side chain
Montijn, R.C.; van Rinsum, J.; van Schagen, F.A.; Klis, F.M.

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Mannoproteins in the walls of mnn9 cells of Saccharomyces cerevisiae were released by laminarinase, and purified by concanavalin A affinity chromatography and ion-exchange chromatography. Carbohydrate analysis revealed that they contained N-acetylglucosamine, mannose, and glucose. An antisera raised against β(1→6)-glucan reacted with four proteins with molecular masses of 66, 100, 155, and 220 kDa, respectively. Recognition by the antisera was competitively inhibited by β(1→6)-glucan, but not by β(1→3)-glucan, mannan, or dextrans (an α(1→6)-glucan). Mild periodate treatment of the wall proteins completely abolished recognition by the antisera. Glucose-containing side chains were isolated and compared with N- and O-carbohydrate side chains. The glucose-containing side chains consisted of about equal amounts of glucose and mannose and some N-acetylglucosamine, and were larger than N-chains. They were, however, not extended N-chains, because after acetolysis, which preferentially cleaves (1→6)-linked glucose, their elution profiles differed strongly. A model is presented of how glucose-containing side chains might anchor mannoproteins into the glucan layer of the cell wall.

Surface proteins in eukaryotic cells undergo various types of covalent modifications with glycans. Proteins that have entered the secretory pathway can be modified by the attachment of N-glycans to asparagine, and O-glycans to serine or threonine. In yeast, the N-linked glycans consist of a chitobiose unit attached to a mannose, which is α-linked to a mannose, which is α-linked to a mannose, which in turn can be modified by a number of covalent modifications with glycans. Proteins that have extended N-linked side chains are predominantly composed of α(1→6)-linked mannose and β(1→6)-linked glucose residues and represent a unique type of carbohydrate chain. We present a model that explains the contribution of these side chains to the attachment of mannoproteins to cell wall glucan.

EXPERIMENTAL PROCEDURES

Materials

Mannose, mannan, phenylmethylsulfonyl fluoride, BSA, jack bean α-mannosidase, and mollusc laminarinase were purchased from Sigma. Laminarin (β(1→3)-glucan) and β-mercaptoethanol were purchased from Fluka AG. Pustulan (β(1→6)-glucan) was obtained from Calbiochem. Bio-Gel P-10 and P-300 and prestained molecular weight standards for Western analysis were from Bio-Rad. Concanavalin A (ConA), Sepahrose and DEAE-Trisacryl were obtained from Pharmacia Biotech Inc. Endo-β-N-acetylglucosaminidase H, peptide N-glycosidase F, and sweet almond β-glucosidase were purchased from Boehringer Mannheim. Anhydrous hydrazine, BCA-protein assay reagent, and goat-antirabbit IgG/HRP peroxidase were obtained from Pierce. All other chemicals were of analytical grade.

Methods

Yeast Strain and Growth—S. cerevisiae LB347-1C (mnn9, MATα) was kindly made available by Dr. L. Ballou (Department of Biochemistry, University of California, Berkeley, CA). Cells were grown at 28°C in YPD medium (1% (w/v) yeast extract, 1% (w/v) Bacto-peptone (Difco), and 3% (w/v) glucose). Isolation and Purification of Glucanase-extractable Proteins—Cell walls isolated from early exponential-phase cells (7) were boiled in 3% (w/v) SDS, 5 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, to remove SDS-extractable mannoproteins. SDS-extracted cell walls were washed six times with 0.1 M sodium acetate, pH 5.5, containing 1 mM phenylmethylsulfonyl fluoride. To isolate glucanase-extractable mannoproteins, washed cell walls were resuspended in the same buffer (1 g in 2 ml), and 0.25 unit of mannanase was added. After incubation at 35°C for 2 h, again 0.25 unit of the enzyme was added followed by an additional incubation of 2 h. The glucanase-extractable mannoproteins were purified by ConA-Sepharose affinity chromatography and DEAE-Trisacryl anion-exchange chromatography (7). Part of the proteins were digested with Endo H for SDS-polyacrylamide gel electrophoresis and Western analysis (Scheme 1). Cell wall proteins, dissolved (8 mg/ml) in water containing 0.2% SDS (w/v) and 100 mM β-mercaptoethanol, were boiled for 5 min. SDS was removed by precipitating the glucanoses in 5 volumes of cold acetone at −20°C for 2 h. The precipitate was evaporated and dissolved in 1 ml of 50 mM KH₂PO₄, pH 5.5, containing 100 mM β-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride. De-N-glycosylation was performed by adding 40 milliunits of Endo H. After incubating at 37°C for 48 h, again 20 milliunits of Endo H were added to the mixture, and the incubation was continued for another 24 h.

Preparation of Neoglycoproteins—Pustulan (a β(1→6)-glucose polymer with an average degree of polymerization of 120) was partly hydrolyzed in 0.1% trifluoroacetic acid at 100°C for 60 min to obtain

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†To whom correspondence should be addressed. Tel: 20-5257843; Fax: 20-5257934.

1 The abbreviations used are: BSA, bovine serum albumin; ConA, concanavalin A; Endo H, endo-β-N-acetylglucosaminidase H; HIPAC, high pH anion-exchange chromatography; PAD, pulsed amperometric detection; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.
Glucomannoproteins in Yeast

Characterization of Polyclonal Antibodies Specific for β(1-6)-Glucomannan—The binding specificity of the antibodies was determined using a combination of indirect ELISA against β(1-3)-glucan-BSA and β(1-6)-glucan-BSA, and indirect competitive ELISA using pustulan (β(1-6)-glucan), laminarin (β(1-3)-glucan), and yeast mannan. For the indirect ELISA, microtiter plates were coated with either β(1-3)-glucan-BSA or β(1-6)-glucan-BSA (100 µl of 10 µg/ml in PBS, pH 7.2). The coated plates were washed four times with PBS and incubated with serial dilutions (up to 500,000) of antibodies diluted in PBS, 3% BSA, for 1 h at 37°C. The plates were washed four times with PBS and incubated with goat-anti-rabbit IgG-peroxidase conjugate. After 1 h at 37°C, the plates were washed as before and developed with a solution of tetramethylbenzidine as chromogenic substrate (10). The reaction was stopped by placing the blots in 50% ethanol. For competitive Western analysis, the proteins were blotted, the blot was blocked with 3% BSA in PBS, washed with PBS, and incubated with antibodies diluted with PBS, 3% BSA (1/25,000). The binding of the antibodies was detected with goat-anti-rabbit IgG-peroxidase using a solution of amidoarylcarbozole as chromogenic substrate (10). The reaction was stopped by placing the blots in 50% ethanol. For competitive Western analysis, the proteins were blotted, the blot was blocked with 3% BSA in PBS, washed with PBS, and incubated with diluted antibodies (up to 500,000). The inhibition of binding to the target antigen was determined using indirect ELISA. Even at a 1/250,000 dilution, the antiserum to β(1-6)-glucan dropped to zero at a serum dilution of 1:100. Inhibition of binding to β(1-6)-glucan at a serum dilution of 1:100 was determined using indirect ELISA. Even at a 1/250,000 dilution, the antiserum to β(1-6)-glucan dropped to zero at a serum dilution of 1:100.

RESULTS

Characterization of Antibodies against Glucomannan—For the detection and characterization of glucomannoproteins, polyclonal antibodies were raised against β(1-6)-glucan. Because carbohydrates are weak immunogens in rabbits, we coupled a partial hydrolysate of pustulan (β(1-6)-glucan), with an average degree of polymerization of 15 glucose residues to bovine serum albumin (BSA) to enhance the immune response. The binding specificity of the antiserum was determined using indirect ELISA. Even at a 1/2,500,000 dilution, the binding of the antiserum to β(1-6)-glucan dropped to zero at a serum dilution of 25,000. In the presence of 23 µg pustulan (= 250 µg glucose equivalents) the binding of β(1-6)-glucan dropped to zero at a serum dilution of 25,000. In the presence of 23 µg pustulan (= 250 µg glucose equivalents) the binding of β(1-6)-glucan dropped to zero at a serum dilution of 25,000.

Analytical Methods—Protein concentrations were determined with the BCA protein assay reagent with bovine serum albumin as a reference protein. Carbohydrate was measured with phenol-sulfuric acid with mannose as a reference (14).
show that the serum has a strong specificity for β(1-6)-glucan.

**Immunological Detection and Characterization of Glucosylated Cell Wall Proteins**—Glucanase-extractable proteins were released from isolated walls by lamarinanase (β(1-3)-glucanase) digestion and purified by ConA-Sepharose affinity chromatography and DEAE-Trisacryl anion-exchange chromatography. Mnn9 cells, which carry truncated N-chains (E), were separated by SDS-PAGE and western analysis, and (ii) to facilitate the purification of glucose-containing chains (7). When purified mannoproteins (Fraction I) were separated by SDS-PAGE and silver-stained (Fig. 2A), several proteins were found with molecular masses of 410, 280, 220, 170, 110, 66, 52, and 30 kDa, respectively. After Endo H digestion, proteins with molecular masses of 390, 250, 195, 135, 110, and 52 kDa were found, showing that most but not all proteins are sensitive to Endo H. In a Western analysis, the β(1-6)-glucan-specific antibodies recognized four proteins with average molecular masses from 220, 155, 100, and 66 kDa (Fig. 2B). Three of the four proteins recognized by the anti-serum appeared to carry Endo H-sensitive N-chains as shown by the shift in molecular masses from 220, 155, and 100 kDa to 200, 145, and 92 kDa, respectively. The 66-kDa protein does not seem to carry Endo H-sensitive N-chains. Table I shows that in Endo H-digested cell wall mannoproteins the amounts of mannose and N-acetylglucosamine had decreased due to the release of N-chains but at the same time the relative amount of glucose had increased indicating that the glucose-containing side chains are insensitive to Endo H.

To confirm that the epitope on these four cell wall proteins indeed consisted of β(1-6)-glucan, they were first treated with periodate. As shown in Fig. 3B, periodate treatment completely abolished the recognition of the antibodies demonstrating the carbohydrate nature of the epitope. Furthermore, the binding of the antibodies could be inhibited competitively by partially hydrolyzed pustulan (Fig. 3D), but not by mann, laminarin, or dextran (Fig. 3, C, E, and F). These results demonstrate a covalent association between four cell wall proteins and β(1-6)-glucan.

**Acetylation of N-chains and Glucose-containing Side Chains**—Both glucose-containing side chains (Fraction III) and N-chains (Fraction II) contained mannose and N-acetylglucosamine although in different ratios (7). As the glucose-containing side chains were also considerably larger than normal N-chains (7) (see also Fig. 4, A and B), an obvious possibility was that they actually represented modified N-chains obtained by the attachment of β(1-6)-linked glucose residues to the core structure of N-chains. To investigate this, N-chains and glucose-containing chains were analyzed by acetylation which selectively cleaves (1→6)-linkages in oligo- and polysaccharides. Prior to acetylation, N-glycanase-released N-chains separated into five main peaks.

**Table 1**

<p>| Sugar composition of glucanase-extracted cell wall proteins in mnn9 cells (Fraction I) |
|---------------------------------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>N-Acetylglucosamine</th>
<th>Glucose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Endo H</td>
<td>2.7</td>
<td>4.6</td>
<td>92.7</td>
</tr>
<tr>
<td>+ Endo H</td>
<td>1.8</td>
<td>11.2</td>
<td>87.0</td>
</tr>
</tbody>
</table>

The glucanase-extracted mannoproteins were purified by ConA-Sepharose affinity chromatography and DEAE-Trisacryl anion-exchange chromatography. The carbohydrate composition of the purified proteins was determined by HPAEC-PAD.
control periodate mannose pustulan laminarin dextran

**FIG. 3.** Characterization of the epitope of the \( \beta(1-6) \)-glucan antiserum on glucanase-extractable cell wall proteins. Western analysis was performed as described in Fig. 2B. In each lane 5 \( \mu \)g of protein were applied. Lane A, control; lane B, after periodate treatment. Lanes C, D, E, and F, immunoblotting was carried out in the presence of yeast mannose, pustulan hydrolysate (\( \beta(1-6) \)-glucan), laminarin (\( \beta(1-3) \)-glucan), and dextran (\( \alpha(1-6) \)-glucan), respectively.

(FIG. 4A). It cannot be excluded that this heterogeneity is due to degradation occurring during digestion of the isolated walls with laminarinase, since the enzyme preparation used contained some \( \alpha \)-mannosidase activity (7). Trimble and Atkinson (16) have, however, shown that some heterogeneity occurs in \( N \)-linked side chains of total cell mannoproteins. Hydrazinolysis released both \( N \)-linked chains and the glucose-containing side chains. The glucose-containing side chains had a considerably higher mass as shown by gel filtration and by HPAEC-PAD (compare Fig. 4, A and B). They were also heterogeneous (Fig. 4B). Their composition was variable with an average molar ratio between mannose and glucose of 1:1. Fig. 4, C and D, show the acetylation products of purified \( N \)-chains and glucose-containing side chains, respectively. Acetylation of \( N \)-chains yielded three peaks (Fig. 4C). Peak I (15%) co-eluted under separation conditions optimal for monosaccharides with mannose. Peak II (59%) co-eluted under various separation conditions with \( \text{Man}(1-2) \)-Man and \( \text{Man}(1-3) \)-Man. Peak III (18%) did not co-elute with any of the reference oligosaccharides and presumably represents the chitobiose core with 4 or 5 mannose residues attached to it (17). Acetylation of the glucose-containing side chains resulted in a completely different picture (Fig. 4D). There was only one major monosaccharide peak representing 72% of the total hexose and consisting of 43% glucose and 57% mannose (peak IV). The remaining minor peaks did not co-elute with reference oligosaccharides under various separation conditions and were not further characterized. Importantly, components similar to mannobioside(s) and to chitobiose-mannosides, as found among the acetylation products of normal \( N \)-chains (Fig. 4C, peaks II and III), were absent. This strongly indicates that the glucose-containing side chain are not derived from normal \( N \)-chains. Since acetylation mainly yielded monosaccharides, this shows that the majority of the glucose and mannose residues in the glucose-containing side chains are (1-6)-linked.

**Enzymatic Degradation of Glucose-containing Side Chains**—

Exo-\( \alpha \)-mannosidase released 46% of the total hexose from the purified glucomannan chains (Fraction III) as monosaccharides (Fig. 5). The released monosaccharide (Fig. 5, peak I) co-eluted with mannose under conditions optimal for separation of different monosaccharides (profile not shown). The release of 46% of the total hexose as mannose by exo-\( \alpha \)-mannosidase in combination with the fact that glucose-containing side chains consist of approximately 50% mannose, indicates that the enzymatic degradation by exo-\( \alpha \)-mannosidase was complete. These results suggest that glucose-containing side chains consists of a core of \( \beta(1-6) \)-glucan extended with one or more \( \alpha(1-6) \)-mannose chains.

The results of exo-\( \beta \)-glucosidase digestion of purified glucose-containing chains were difficult to interpret because the enzyme preparation was contaminated with \( \alpha \)-mannosidase activity capable of degrading \( N \)-chains (not shown). Indeed, the released monosaccharides consisted of 44% glucose and 56% mannose under separation conditions optimal for monosacchara-
that glucose-containing chains represent a novel type of carbohydrate chain on cell wall mannanproteins. It seems likely that at least part of the synthesis of the β(1-6)-glucan side chains takes place intracellularly. It is tempting to speculate that KRE5 and KRE6 are involved in the synthesis of this side chain, since both genes code for proteins involved in the synthesis of β(1-6)-glucan and are located in the endoplasmic reticum and Golgi, respectively (18).

Although many structural features of yeast cell wall components are known, not much information is available on the architecture of the cell wall (for a recent review see Ref. 19). One of the major questions is how mannanproteins are anchored in the wall. The ability of β(1-3)-glucanase to liberate mannanproteins from cell walls suggests that they are closely associated with cell wall glucan (4). We suggest the following model for the anchoring of glucomannoproteins in the wall. According to this model, glucomannoproteins contain besides N- and O-linked chains, glucose-containing side chains linked to the protein moiety of the mannanprotein. In vivo, the glucose-containing side chains, which contain β(1-6)-linked glucose residues, might be extended with β(1-3)-linked glucose chains, which are removed during the isolation of the glucomannoproteins. These β(1-3)-linked glucose chains might interweave with the β(1-3)-glucan fibrils in the cell wall thereby anchoring the glucomannoproteins.

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