Glucomannoproteins in the cell wall of Saccharomyces cerevisiae contain a novel type of carbohydrate side chain
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Mannoproteins in the walls of *mn9* 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 antisemur raised against β(1-6)-glucan reacted with four proteins with molecular masses of 66, 100, 155, and 220 kDa, respectively. Recognition by the antisemur was competitively inhibited by β(1-6)-glucan, but not by β(1-3)-glucan, mannan, or dextran (an α(1-6)-glucan). Mild periodate treatment of the wall proteins completely abolished recognition by the antisemur. 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 walls, they contained glucosamine, mannose, and galactose residues. An antiserum raised against p(l-6)-

EXPERIMENTAL PROCEDURES

Materials

Mannose, mannnitol, phenylmethylsulfonyl fluoride, BSA, jack bean α-mannosidase, and molluc 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), Sepharose 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-anti-rabbit IgG/horseradish peroxidase were obtained from Pierce. All other chemicals were of analytical grade.

Methods

Yeast Strain and Growth—*S. cerevisiae* LB347-1C (*mn9, MAtα*) was kindly made available by Dr L. Balou (Department of Biochemistry, University of California, Berkeley, CA). Cells were grown at 28 °C in YPD medium (1% (w/v) yeast extract (Life Technologies, Inc.), 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 2% (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 mannnase laminarinase 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, Fraction I). 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 glucanoproteins in 9 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 incubation at 37 °C for 48 h, again 20 milliunits of Endo H were added to the mixture, and the incubation continued for another 24 h.

Preparation of Neoglycoproteins—Pustulan (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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The abbreviations used are: BSA, bovine serum albumin; ConA, concanavalin A; Endo H, endo-β-N-acetylglucosaminidase H; IPACE, high pH anion-exchange chromatography; PAD, pulsed amperometric detection; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.
the inhibitors at the desired concentrations. The plates were developed as described.

**Immunoblotting**—Electrophoresis was performed on linear gradient (2.2–20%) polyacrylamide gels according to Laemmli (11). Proteins were either stained by the silver staining method as described by De Nobl et al. (12) or electrophoretically transferred to an Immobilon polyvinylidene fluoride membrane for Western analysis. The inhibitors at the desired concentrations. The plates were developed as described.

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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 laminarinase (β(1-3)-glucan) digestion and purified by ConA-Sepharose affinity chromatography and DEAE-Trisacryl anion-exchange chromatography. Mnn9 cells, which carry truncated N-chains (E), were used for two reasons: (i) to obtain discrete protein bands were used for two reasons: (i) to obtain discrete protein bands for SDS-PAGE and for Western analysis, and (ii) to facilitate the purification of glucose-containing chains (7). When purified mannoproteins (Fraction 1) 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 of 220, 155, 100, and 66 kDa (Fig. 2B). These proteins probably correspond with the 220-, 155-, 110-, and 66-kDa bands in the silver-stained gel (Fig. 2A). Three of the four proteins recognized by the anti-serum appeared to carry Endo H-sensitive N-chains as shown by the reduction of the 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 mannan, 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-acetylgalactosamine 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...
control periodate mannans pustulan laminarin dextran

FIG. 3. Characterization of the epitope of the β(1-6)-glucan antiserum on glucanase-extractable cell wall proteins. Western analysis was performed as described in Fig. 2B. In each lane 5 µg 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 mannan, pustulan hydrolysate (β(1-6)-glucan), laminarin (β(1-3)-glucan), and dextran (α(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 α-mannosidase activity (17). 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 Manα(1-2)-Man and Manα(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 mannosides (s) and to chitobiose-mannobiosides, 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 chains 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-α-mannosidase released 46% of the total hexose from the purified glucosmannan 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-α-mannosidase in combination with the fact that glucose-containing side chains consist of approximately 50% mannose, indicates that the enzymatic degradation by exo-α-mannosidase was complete. These results suggest that glucose-containing side chains consists of a core of β(1-6)-glucan extended with one or more α(1-6)-mannose chains.

The results of exo-β-glucosidase digestion of purified glucose-containing chains were difficult to interpret because the enzyme preparation was contaminated with α-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 monosacchar-
DISCUSSION

The immunological data show that the cell wall of S. cerevisiae mnn9 cells contains four glycosylated proteins carrying a novel type of carbohydrate side chain characterized by the presence of β(1→6)-linked glucose residues. These proteins represent a specific subset of the total number of the glucanase-extractable proteins. Because we isolated these β(1→6)-glycosylated cell wall proteins after digestion of the cell wall with β(1→3)-glucanase, possible branching of this β(1→6)-glucan side chain with β(1→3)-glucan would have been digested and would therefore not have been detected.

Glucose-containing chains cannot be released from cell wall glucomannoproteins by β-elimination (7) (data not shown) indicating that they are not O-linked to serine or threonine. They can, however, be freed by a harsher alkali treatment (7) or hydrazinolysis (this paper). The similar molar ratio of glucose and mannose in the chains released by the two methods (1:1), as well as the fact that similar products were obtained by chemical or enzymatic degradation, indicates that both methods released the same kind of chain. Acetylation of glucose-containing side chains indicated that the majority of glucose and mannose residues are 1,6-linked and that only a few branch points are present. In combination with the results obtained by exo-α-mannosidase digestion, this indicates that most or all mannose residues are α(1→6)-linked. Similarly, exo-β-glucosidase digestion indicated that at least part of the glucose residues are β-linked. The limited release of glucose by β-glucosidase digestion might be due to the inaccessibility of glucose residues. Summarizing, the glucose-containing chains differ from known N-chains with respect to their resistance to peptide N-glycosidase F and Endo H (7), their composition, their behavior on HPAEC, and with respect to the reaction products of chemical and enzymatic degradation. It is therefore concluded that glucose-containing chains represent a novel type of carbohydrate chain on cell wall mannoproteins. 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 reticulum 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 mannoproteins are anchored in the wall. The ability of β(1→3)-glucanase to liberate mannoproteins 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 mannoprotein. In vivo, the glucose-contain ing 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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