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Glucosylation of chimeric proteins in the cell wall of *Saccharomyces cerevisiae*

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Abstract

Extension of a reporter protein with the carboxyterminal thirty amino acids of the cell wall mannoprotein α-agglutinin of *Saccharomyces cerevisiae* resulted in incorporation of the chimeric protein in the cell wall. By Western analysis it was shown that the incorporated protein contained β-1,6-glucan similar to endogenous cell wall proteins, whereas excreted reporter protein was not glucosylated. This suggests that β-1,6-glucan is involved in anchoring mannoproteins in the cell wall.

Key words: Glycosylation; Glucan; Mannoprotein; GPI-anchor; α-Agglutinin; α-Galactosidase; Yeast

1. Introduction

The cell wall of *Saccharomyces cerevisiae* consists of a glucan layer covered by a layer of mannoproteins. Mannoproteins carry large, branched mannan polysaccharides, *N*-glycosidically linked to asparagine residues, and short oligomannosides, *O*-glycosidically linked to serine or threonine. Although some mannoproteins can be extracted from cell walls by detergent, most mannoproteins can only be released by digesting walls with a β-1,3-glucanase, indicating that they are tightly associated with the glucan layer (see [1] for recent review about the cell wall).

We recently demonstrated that several β-1,3-glucanase-extractable wall proteins are covalently linked to a β-1,6-glucan [2,3]. This raised the question whether this type of side-chain is involved in anchoring glucanase-extractable mannoproteins in the cell wall. To answer this question, we investigated the incorporation of α-agglutinin, the sexual adhesion protein in the cell walls of *MATα* cells. The N-terminal part of this protein is involved in sexual adhesion [4]. The C-terminal half consists for about 50% of serine and threonine [5], suggesting that it might function as a spacer domain due to a high density of *O*-linked oligomannosides [6]. At the C-terminus, a functional addition signal for a glycosylphosphatidylinositol (GPI) membrane anchor is present [4]. We show here that a reporter protein extended with the carboxyterminal thirty amino acids of α-agglutinin is incorporated in the cell wall and contains β-1,6-glucan. In contrast, reporter protein that is recovered from the culture fluid and apparently is secreted is not glucosylated, suggesting that the attachment of a β-1,6-glucan side-chain plays a role in anchoring β-1,3-glucanase-extractable mannoproteins in the cell wall.

2. Materials and methods

Saccharomyces cerevisiae BJ2168 (*MATα, leu2, trp1, ura3–52, prb1–112, pep4–3, prc1–407 gal2*) was obtained from the Yeast Genetic Stock Centre (Berkeley, CA, USA). Cells were transformed with plasmids encoding the fusion proteins depicted in Fig. 1. Plasmid pSY13 [7] encodes αgal, plasmid pPGA1 [7] encodes the chimeric protein αgal-320AGα1, and plasmid pPGA2 encodes the chimeric protein αgal-30AGα1. pPGA2 was constructed using pSY13 and the AGα1 gene encoding α-agglutinin [5], kindly provided by Dr. J. Kurjan. The *Sty*I restriction site at position 1143 in the coding sequence of αgal in pSY13 was ligated to the *Bsp*HI restriction site at position 1,859 in the coding sequence of the AGα1 gene. To obtain an in frame fusion, the *Sty*I and BspH1 overhanging ends were filled in with Klenow DNA polymerase. The *Hind*III site in the 3′ untranslated part of the AGα1 gene was ligated to the *Hind*III site preceding the PGK terminator in pSY13.

α-Galactosidase activity of transformants was detected on plates containing 5-bromo-4-chloro-3-indolyl-α-D-galactose (X-α-Gal) and was quantified using *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) as described previously [7]. Cultures were grown to an $OD_{530\ nm}$ of 2.0. Cell walls were isolated as in [2], boiled in the presence of SDS, EDTA and β-mercaptoethanol to obtain detergent extracts as in [7] and subsequently digested with laminarinase to obtain β-1,3-glucanase extracts as in [2]. Proteins present in the culture fluid were precipitated using deoxycholate [8]. Western analysis was carried out as described previously [3], except that enhanced chemiluminescence (ECL) detection was used according to the manufacturer's protocol (Amersham International, Little Chalfont, Buckinghamshire, UK). Fractions for Western analysis were equivalent to 250 μl of culture fluid, or to the detergent or β-1,3-glucanase extract of 1 mg cell walls (wet weight). α-Galactosidase antiserum was raised in rabbits using purified α-galactosidase from guar (kindly provided by Dr. J. Verbakel, Unilever, Vlaardingen, The Netherlands) and was purified by adsorption on acetone powder of BJ2168 cells [9]. β-1,6-Glucan antiserum was raised in rabbits using BSA-pustulan glycoconjugates [3] and was purified by affinity chromatography on a pustulan-Sepharose column.

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Abbreviations: GPI-anchor: glycosylphosphatidylinositol anchor; X-α-Gal: 5-bromo-4-chloro-3-indolyl-α-D-galactose; *p*NPG: *p*-nitrophenyl-α-D-galactopyranoside; SDS: sodium dodecyl sulfate; EDTA: ethylenediaminetetraacetic acid

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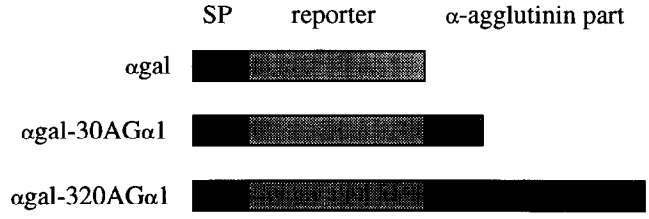

Fig. 1. Schematic representation of the proteins αgal, αgal-30AGα1 and αgal-320AGα1. SP: signal peptide of yeast invertase; reporter: guar α-galactosidase; α-agglutinin part: 30 carboxyterminal amino acids (αgal-30AGα1) or 320 carboxyterminal amino-acids (αgal-320AGα1).

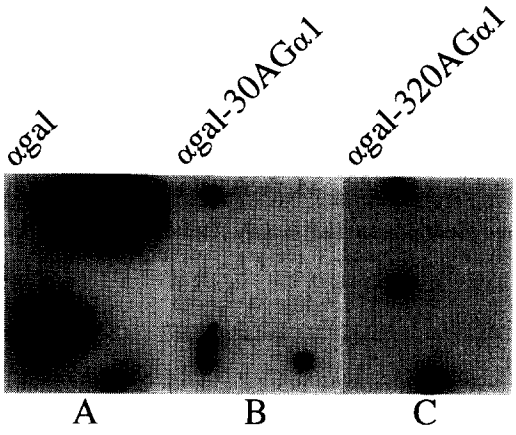

Fig. 2. Colonies of BJ2168 cells expressing αgal (panel a), αgal-30AGα1 (panel b) or αgal-320AGα1 (panel c) on medium containing X-α-Gal.

3. Results

The fusion protein αgal consists of guar α-galactosidase preceded by the signal sequence of yeast invertase. The chimeric proteins αgal-30AGα1 and αgal-320AGα1 were constructed by C-terminal extension of αgal with carboxyterminal parts of α-agglutinin (Fig. 1). Fig. 2 shows that colonies of cells expressing αgal formed large, faint-blue halos in the presence of the chromogenic substrate X-α-Gal due to secretion of α-galactosidase into the medium. On the other hand, colonies of cells expressing either αgal-30AGα1 or αgal-320AGα1 became dark-blue and formed only very small halos, indicating that both chimeric proteins were largely retained at the cell surface. Assay of α-galactosidase activity with the chromogenic substrate *p*NPG confirmed that αgal was almost entirely secreted into the medium, whereas the activity in cells expressing either αgal-30AGα1 or αgal-320AGα1 was mainly associated with the cell walls (Table 1). Western analysis of components of the growth medium of αgal-cells with α-galactosidase antiserum showed the presence of a predominant form of α-galactosidase with an M_r of 40 kDa (Fig. 3, lane 1) as expected from the sequence data [10]. A considerable part of the cell wall protein αgal-30AGα1 could only be released by digesting the walls with a β-1,3-glucanase and had an M_r of 50 kDa (Fig. 3, lane 3). Material released by detergent extraction had a slightly smaller M_r of 45 kDa (Fig. 3, lane 2), suggesting that it is either a precursor or a degradation product of the glucanase-extractable form. In contrast, the chimeric protein αgal-320AGα1 was almost entirely recovered in the β-1,3-glucanase extract of isolated walls. The most abundant form had an M_r of 350 kDa (Fig. 3, lane 5). A ladder of products of lower molecular mass probably representing degradation products was also present. The detergent extract contained a very faint band of 75 kDa (Fig. 3, lane 4). Control experiments showed that no α-galactosidase activity was detected biochemically or immunologically in untransformed cells (not shown).

To test whether the chimeric cell wall proteins αgal-30AGα1 and αgal-320AGα1 contained β-1,6-glucan, cell wall extracts were subjected to Western analysis with β-1,6-glucan specific antiserum. In the β-1,3-glucanase extract of walls of untransformed cells, four proteins were detected of 205, 145, 80 and 55 kDa (Fig. 4, lane 2). In the β-1,3 glucanase extract of cells that expressed the small chimeric protein, an additional protein was detected with an M_r of 50 kDa (Fig. 4, lane 1), corresponding with the β-1,3-glucanase-extractable form of this protein (Fig. 3, lane 3). Likewise, in the β-1,3-glucanase extract of cells expressing the large chimeric protein, an additional protein was detected with an M_r of 350 kDa (Fig. 4, lane 3). However, the antiserum did not bind to secreted αgal (Fig. 4, lane 7), nor to the detergent-extractable 45-kDa form of αgal-30AGa or the faint 75-kDa band in the detergent extract of αgal-320AGα1 cell walls. Interestingly, the detergent extracts of all cell types contained some high molecular weight material that

Table 1
Distribution of α-galactosidase activity in BJ2168 cells expressing αgal, αgal-30AGαl, or αgal-320AGαl

Expressed protein	α-Galactosidase activity (U/g fresh weight cells)		
	Growth medium	Intact cells	Isolated cell walls
αgal	53.1 ± 6.5 (n = 5)	0.13 ± 0.09 (n = 3)	0.06 ± 0.03 (n = 5)
αgal-30AGαl	0.4 ± 0.1 (n = 5)	6.9 ± 2.2 (n = 3)	9.1 ± 1.3 (n = 5)
αgal-320AGαl	4.6 ± 0.4 (n = 5)	28.0 ± 6.2 (n = 3)	19.3 ± 3.4 (n = 5)

One unit of activity corresponds to the hydrolysis of 1 μmol *p*NPG per min at 37°C, pH 4.5. Figures are means ± S.E.M. with the number of independant transformants tested in parentheses.

hardly entered the gel (Fig. 4, lanes 4, 5 and 6). Competition experiments confirmed that the antiserum specifically bound to β-1,6-glucan. Addition of pustulan (β-1,6-glucan) abolished the reactivity of the proteins to the antiserum, but addition of laminarin (β-1,3-glucan) or mannan had no effect. In addition, periodate, which destroys β-1,6-glucan but has no effect on β-1,3-glucan, abolished the reactivity of the proteins to the antiserum (not shown). These results demonstrate that the chimeric glucanase-extractable wall proteins αgal-30AGα1 and αgal-320AGα1 contain β-1,6-glucan, whereas secreted αgal is not glucosylated.

4. Discussion

We show here that fusion of a carboxyterminal part of α-agglutinin as short as thirty amino acids to a reporter enzyme leads to incorporation of the chimeric protein in the cell wall. Wojciechowitcz et al. [4] have demonstrated that deletion of the carboxyterminal fifteen amino acids of α-agglutinin allows efficient secretion of biologically active α-agglutinin. It seems therefore likely that the addition of a terminal GPI-anchor to α-agglutinin is essential for incorporation of the adhesion molecule in the cell wall. However, several GPI-anchored proteins are plasma membrane-linked [11,12], suggesting that addition of a GPI-anchor is in itself not sufficient for cell wall incorporation. Since the carboxyterminal thirty amino acids of α-agglutinin do not

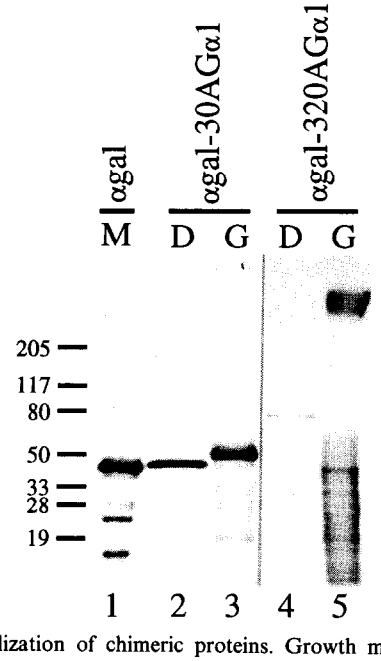

Fig. 3. Localization of chimeric proteins. Growth medium (M) of BJ2168 cells expressing αgal, and detergent extracts (D) or β-1,3-glucanase extracts (G) of cell walls of BJ2168 cells expressing αgal-30AGα1 or αgal-320AGα1 were subjected to Western analysis with α-galactosidase antiserum. Marker sizes are indicated in kilodaltons.

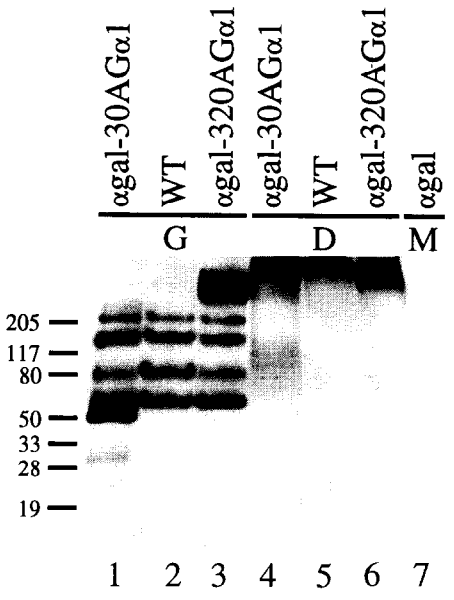

Fig. 4. Glucanase-extractable proteins contain β-1,6-glucan. Growth medium (M) of BJ2168 cells expressing αgal, and detergent extracts (D) or β-1,3-glucanase extracts (G) of cell walls of BJ2168 cells (WT) or BJ2168 cells expressing αgal-30AGα1 or αgal-320AGα1 were subjected to Western analysis with β-1,6-glucan antiserum. Marker sizes are indicated in kilodaltons.

contain many serine and threonine residues and lack potential *N*-glycosylation sites [5], extensive mannosylation cannot play a role in binding. We show here that chimeric glucanase-extractable wall proteins consisting of a reporter enzyme and a carboxyterminal part of α-agglutinin contain β-1,6-glucan similar to endogenous wall proteins. In contrast, secreted reporter enzyme recovered from the growth medium is not glucosylated. This suggests that the attachment of β-1,6-glucan plays a role in anchoring glucanase-extractable mannoproteins in the glucan layer of the cell wall. This β-1,6-glucan might be attached to a cell wall-specific type of GPI-anchor, as was recently hypothesised by De Nobel and Lipke [13]. According to this view, glucose should be absent from the carbohydrate part of GPI-anchors of plasma membrane-bound proteins. Indeed, so far no glucose has been detected in these structures [14,15].

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