Selective elongation of the oligosaccharide attached to the second potential glycosylation site of yeast exoglucanase: effects on the activity and properties of the enzyme

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Three exoglucanases (Exgs), Exgla, Exglb and Exg325, are secreted by Saccharomyces cerevisiae cells. They share a common protein portion with two potential glycosylation sites (sequons) but differ in the amount of N-linked carbohydrate [Basco, R. D., Muñoz, M. D., Hernández, L. M., Vázquez de Aldana, C. and Larriba, G. (1993) Yeast 9, 221–234]. Exglb contains two short oligosaccharides attached to asparagines (Asn) 165 and 325 of the primary translation product [Hernández, L. M., Olivero, I., Alvarado, E. and Larriba, G. (1992) Biochemistry 31, 9823–9831]. Exg325 carries a single, short oligosaccharide bound to Asn325 whereas Exgla has at least one large oligosaccharide, since it has not been produced by mutant mnt9. To address the question of the origin of Exgla, both sequons were individually mutated by substituting Gln for Asn. An Exgla-like isoenzyme was still secreted by mutant Exg165 but not by mutant Exg325.

Additional studies on sequential deglycosylation of Exgla with endo-β-N-acetylglucosaminidase H (endo H), the susceptibility of both oligosaccharides to the endoglycosidase, and analysis of the presence of GlcNAc at both asparagine residues after total deglycosylation with endo H, indicated that Exgla contained two oligosaccharides, a short one bound to Asn165 and a large one bound to Asn325, and, accordingly, originated from Exglb. The elongation of the second oligosaccharide did not result in a higher stability towards thermal inactivation or unfolding, or in an increased resistance to proteases as compared with Exglb; however, the affinity of the enzyme towards laminarin decreased by 50%. This site-specific elongation occurred in the oligosaccharide that was less susceptible to endo H, indicating that these properties are determined by different conformational constraints.

INTRODUCTION

Glycosylation is a major covalent modification that modulates the structure and/or the function of many proteins in eukaryotes. Of the several types of protein glycosylation, the addition of N-oligosaccharides to Asn residues has been studied in great detail and has been shown to proceed by a pathway that has been highly conserved through evolution (Kornfeld and Kornfeld, 1985). Studies in Saccharomyces cerevisiae have yielded invaluable information on some steps of this pathway, especially on the assembly of the oligosaccharide–lipid carrier (dolichol-PP), its transfer to the protein and on the trimming and elongation reactions that take place during the transport of the glycoconjugate to the cell surface (Kukuruzinska et al., 1987; Tanner and Lehle, 1987; Herscovics and Orlean, 1993). However, information concerning the structural principles that govern the frequency of glycosylation of different sequons and/or the factors that determine the extent of maturation or elongation of each carbohydrate chain is rather scarce and conflicting. As noted, relatively few N-linked sites have been characterized (Herscovics and Orlean, 1993).

The exoglucanase (Exg) system from S. cerevisiae is an excellent model for this kind of study. The primary translation product of the S. cerevisiae EXG1 gene can be differentially glycosylated during its transit to the cell surface to yield three isoenzymes formerly named ExgI, ExgII and ExgII1/2; here renamed as Exgla, Exglb and Exg325. Exgla and Exglb represent about 10% and 90% respectively of the activity secreted into the culture medium by exponentially growing cells (Ramírez et al., 1989a).

Exg325 is a minor product synthesized during the late exponential and stationary phases of growth when the efficiency of glycosylation is reduced. Under some circumstances, an additional glycoform, Exg165 (previously ExgII1/2'), is also produced (Basco et al., 1993).

Figure 1 shows the primary structure of the several glycoforms, including that deduced for Exgla in the present work. Exglb contains 12% carbohydrate distributed into two short oligosaccharides, each consisting of a regular inner core whose outer chain is reduced to two or three residues of mannose (Ramírez et al., 1989b; Hernández et al., 1992). They are attached to both

Figure 1  Primary structure of the Exgl glycoforms

Wavy line, Exgl polypeptide; solid line, inner core; broken line, outer chain extension. N(165) and N(325) indicate the positions of the Asn of the first and the second sequons respectively. The structure of Exgl stems from the present study. The new and old nomenclature (the last one in parentheses) is indicated under each glycoform. ExgTM, the enzyme secreted in the presence of tunicamycin.

Abbreviations used: Exg, exoglucanase; endo H, endo-β-N-acetylglucosaminidase H; p-NPG p-nitrophenyl-β-D-glucopyranoside; PMSF, phenylmethylsulphonyl fluoride.

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potential glycosylation sites (Asn145 and Asn149) present in the polypeptide (Vázquez de Aldana et al., 1991; Basco et al., 1993). Exg₁ and Exg₂ carry a single oligosaccharide attached to the second and the first glycosylation sites respectively (Basco et al., 1993). Exg₁A contains 30–40% carbohydrate, smears in SDS/acylamide gels as other heavily glycosylated yeast glycoproteins (i.e. invertase, acid phosphatase), and its synthesis is prevented in mutant mmr; accordingly, it was suggested that this isoenzyme arose by elongation of the first, the second or both short oligosaccharides present in Exg₁b (Ramírez et al., 1990). However, the more recent detection of Exg₁₂ and Exg₁₄ in the supernatant fluids of cell cultures (Basco et al., 1993) adds two new candidates to the possible precursors of Exg₁A. The present study has been undertaken to investigate these five possibilities in the hope that it can provide some information on the constraints that govern the elongation of oligosaccharides. Here, we demonstrate that Exg₁A arises from Exg₁b by elongation of the oligosaccharide bound to the second glycosylation site (AsnⅠ). The resulting enzyme, Exg₁A, shows identical stability to thermal inactivation and resistance to proteases as its precursor (Exg₁b). However, it exhibits a decrease in its affinity towards substrates of high (laminarin) but not low (p-nitrophenyl-β-D-glucopyranoside; p-NPG) molecular mass.

MATERIALS AND METHODS

Strains, plasmids, growth conditions and materials

*S. cerevisiae* wild-type strain X2180 1A (MATα gal2) and mutant CV55 (MATα exgⅠ ura3–52 ino1–1 can1), a strain in which the structural *EXGⅠ* gene has been deleted, have been described before (Vázquez de Aldana et al., 1991; Basco et al., 1993). Yeast cells were maintained in YEPD medium (1% yeast extract/2% bactopeptone/5% glucose/2% agar). For the production and purification of external Exg, cells were grown in liquid minimal medium (Olivero et al., 1985) with the appropriate supplement of growth factors. The multicopy plasmid pRN14, which carries the *EXGⅠ* gene, has been described before (Nebreda et al., 1986). Single-stranded DNA templates for sequencing were isolated from MV1190 cells (Bio-Rad) carrying the appropriate plasmids after infection with M13K7 helper phage (Messing, 1983).

Endo-β-N-acetylgalactosaminidase (endo H) was a gift from Dr. F. Maley. Protease Type XIV from *Streptomyces griseus* and trypsin were from Sigma. Other chemicals were obtained as described (Hernández et al., 1986, 1992; Larriba et al., 1988; Ramírez et al., 1989a,b).

Recombinant DNAs and generation of site-specific mutants

The *EXGⅠ* gene was subcloned by placing the HindIII–HindIII fragment into an HindIII site of Bluescript KS+ vector (Stratagene, San Diego, CA, U.S.A.) and the resulting plasmid was designated pCV5. pRS316-2 plasmid was constructed by elimination of KpnI, Sall and Xbal restriction sites into pRS316 vector (Sikorski and Hieter, 1989), since these sites are present in the *EXGⅠ* gene. Site-specific mutants in the first, the second and both N-glycosylation sites of the *EXGⅠ* gene were generated as previously described (Basco et al., 1993). Then, the resultant plasmids were digested with Sall/Xbal (site 1) or KpnI/Nael (site 2) and fragments were subcloned in a plasmid (pRB1) obtained by inserting the BamH1/Clal fragment of *EXGⅠ* from pCV5 into pRS316-2. These constructions were named pRB2 (site 1 mutated), pRB3 (site 2 mutated) and pRB4 (sites 1 and 2 mutated).

Plasmid preparation, agarose gel electrophoresis and DNA transformation of *Escherichia coli* and *S. cerevisiae* were as described before (Ito et al., 1983; Rose et al., 1989; Sambrook et al., 1989).

Ion-exchange chromatography

Conventional ion-exchange chromatography was performed as previously described (Ramírez et al., 1989a). However, a 0–0.35 M NaCl gradient (instead of 0–0.5 M) was used for the separation of ExgⅠb and Exg₁₂. For ion-exchange chromatography at high pressure (h.p.l.c.) dialysed samples (20–100 μl) were applied to an ion-exchange (Bio-Gel TSK DEAE-5-PW) column (75 mm × 7.5 mm). Elution was performed as indicated at a flow rate of 0.5 ml/min and a pressure of 4 bar (1 bar = 10⁶ Pa).

Spectroscopic measurements

An Hitachi–Perkin-Elmer fluorescence spectrophotometer model 650-40 equipped with register and thermoprogrammer were used for spectroscopic measurements. The thermal, unfolding transition of ExgⅠ and ExgⅡ was monitored by fluorescence at 344 nm (excitation at 280 nm). Slit widths of 5 and 10 nm were used for excitation and emission respectively. The heating rate was 4°C/5 min. Samples were resuspended in 25 mM acetate buffer, pH 5.2, supplemented with 20% glycerol to optimize the measurements.

Kinetic measurements

Samples in acetate/glycerol buffer (see above) were incubated for 5 min at the indicated temperatures. Then they were diluted 50-fold in acetate buffer pre-warmed at 30°C. Residual activity was assayed at this temperature against p-NPG. Controls kept at 30°C were assayed in parallel. The protein concentration was about 0.2 mg/ml.

Digestion with proteases

Purified ExgⅠa and ExgⅠb (1 μg of protein) were incubated with protease Type XIV from *S. griseus* (0.12 unit) or trypsin (0.44 unit) in 50 mM Tris/HCl, pH 7 (final volume 30 μl). Incubation times were 0, 5, 10, 20 and 30 min for the former protease and 0, 5, 10, 30, 45 and 60 min for the latter. Reactions were stopped by the addition of 220 μl of 100 mM acetate buffer, pH 5.2, containing the following protease inhibitors: 2 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM EDTA, 2 mM EGTA, 1 mM leupeptin, 1 mM pepstatin and 1 μM aprotinin. The residual Exg activity was tested using p-NPG as a substrate.

Other methods

Enzymic assays were carried out as described by Ramírez et al. (1989a) using either p-NPG or laminarin as the substrate. Glucose liberated from laminarin was determined with glucose oxidase coupled to peroxidase (Gascón and Lampen, 1968). Units of activity were μmol of p-nitrophenol released/h at 30°C. Purification of ExgⅠa and ExgⅠb, gel filtration through Sephacryl S-200 and SDS/PAGE have been described elsewhere (Ramírez et al., 1989a). After electrophoresis samples were stained with silver (Morrisay, 1981). Standard deglycosylation reactions (5 m-units of endo H and about 15 units of exoglocanase in a final vol. of 280 μl) were performed in 50 mM citrate/phosphate buffer, pH 5.6, for 5 h in the presence of PMSF (1 mM) and pepstatin A (20 μM).
RESULTS AND DISCUSSION

Analysis of the Exg complement secreted by wild-type and site-directed glycosylation mutants

The centromeric vector pRS316-2 carrying wild-type EXG1 or its site-directed mutant counterparts was used to transform S. cerevisiae CV55. In the site-directed mutants, glutamines were substituted for asparagines in the first (pRB2, mutant Exg165), the second (pRB3, mutant Exg325) or both (pRB4, mutant Exg165,325) glycosylation sites. The transformed strains were grown in minimal K medium and culture fluids, harvested in the exponential phase, concentrated by ultrafiltration and analysed by standard ion-exchange chromatography (DEAE-Bio-Gel A). As shown in Figure 2(a), strains containing the wild-type gene generated the typical exoglucanase profile, i.e. a small and broad peak (Exgl) followed by a larger and sharper peak (Exglb). The major exoglucanases from site-directed mutants eluted as expected, i.e. Exg165 (secreted by mutant Exg165) eluted at a lower salt concentration than Exg165 (secreted by mutant Exg325), although this chromatographic system was less able to resolve the activity than h.p.l.c. used in previous studies (Basco et al., 1993; see below). More importantly, whereas an Exgl-like enzyme was present in mutant Exg165 (Figure 2c), no activity could be detected in this region of the eluent in samples from mutant Exg325 (Figure 2b). This result indicated that only the second oligosaccharide was elongated in the site-directed mutants. As expected, the double mutant secreted a single form that eluted with the enzyme produced in the presence of tunicamycin (results not shown).

Time course of deglycosylation of Exgl

Since the second oligosaccharide was only present in Exg325, it seemed reasonable to consider this isoenzyme, in addition to Exglb, as a potential candidate for a precursor of Exgl. In order to answer this question, we subjected Exgl to a time-course deglycosylation with endo H and analysed the products by using SDS/PAGE. In the case of Exglb, this procedure demonstrated the presence of an intermediate, a fact indicative of the presence of two N-oligosaccharides attached to the protein. As shown in Figure 3, no intermediate (or only traces of it) was observed during the conversion of Exgl into the deglycosylated product. This result first suggested that Exglb had a single sugar residue and, accordingly, it originated from Exg325. However, the same result would have been obtained if Exglb derived from Exglb, provided that only one Exglb oligosaccharide had been elongated and that the short counterpart was more susceptible to endo H. Clearly, the methodology used was unable to detect an average decrease of 3 kDa in the heterogeneous population of Exglb molecules.

We have reported preliminary evidence indicating that the first sugar residue of Exglb is more susceptible to endo H whereas the second mask more negatively charged residues, a fact which allows the separation and identification of two ionic intermediates carrying a single oligosaccharide in either site (and GlcNAc in the other) (Basco et al., 1993). Additional evidence for this differential susceptibility to endo H came from time-course deglycosylation kinetics of the major glycoforms secreted by mutants Exg165 and Exg325. As shown in Figure 4, Exg325, which carries the oligosaccharide bound to the second sequon, was deglycosylated at a significantly lower rate than Exg165. Assuming that elongation of the second residue did not alter significantly its susceptibility to endo H, we concluded that the approach...
described in Figure 3 does not answer unequivocally the question of the origin of Exgl.  

Characterization of the in vitro deglycosylated Exgl

In order to definitively answer the question of the origin of Exgl, we analysed the deglycosylation products of Exgl from wild-type and Exg165 in a TSK DEAE-5-PW column chromatography (h.p.l.c.), which could discriminate between molecules carrying a single GlcNAc residue attached to either one or both glycosylation sites and their counterparts lacking the amino sugar. A summary of the results obtained during the calibration of the TSK DEAE column with authentic standards is shown in Figure 5(c).

Fractions 36-48 (Figure 2a) and 40-48 (Figure 2c) were pooled, and the enzymes further purified through a Sephacryl S-200 column. Exgl from wild-type and Exg165 mutant were then treated with endo H, and the deglycosylated products analysed by h.p.l.c. (TSK DEAE column). As shown in Figure 5, deglycosylated Exgl (a) from wild-type co-eluted with deglycosylated Exglb one fraction before the deglycosylated Exgl from mutant Exg165 (b) which, in turn, co-eluted with the deglycosylated major Exgl secreted by the same mutant. The retention times corresponded to standards c and d from Figure 5(c) respectively, which carried GlcNAc residues attached to both (form c) or only the second (form d) glycosylation sites. These results strongly suggested that Exgl arose by elongation of the second oligosaccharide of Exglb.

Table 1 Comparison of the \( K_a \) values of purified Exgs I and II and their deglycosylated products in vitro

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Endo H treatment</th>
<th>Laminarin (mg/ml)</th>
<th>p-NPG (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exgl</td>
<td>-</td>
<td>16.0 ± 2.0</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Exgl</td>
<td>+</td>
<td>6.1 ± 0.7</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>ExglII</td>
<td>-</td>
<td>8.0 ± 0.5</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>ExglII</td>
<td>+</td>
<td>7.9 ± 0.4</td>
<td>4.1 ± 0.2</td>
</tr>
</tbody>
</table>

Effect of elongation of the second oligosaccharide on the properties of the enzyme

We first compared the affinities of Exgl, Exglb and their endo H-deglycosylated products towards two different substrates, laminarin and p-NPG. As shown in Table 1, Exgl exhibited half of the affinity for laminarin that Exglb did, in spite of the fact
that both isoenzymes showed the same affinity towards p-NPG. This behaviour should be attributed to the longer sugar chains present in the second glycosylation site of Exgl, which might sterically hinder the binding of the enzyme to substrates of high (laminarin) but not low (p-NPG) molecular mass. In contrast, the short sugar chains of both Exglb oligosaccharides did not appear to have any effect on its affinity towards laminarin (nor towards p-NPG), since the $K_m$ was not modified by treatment of the enzyme with endo H.

Thermal transition curves, determined by enzyme activity, were identical for both isoenzymes and indicated that they have a half-life of 5 min at 62.5 °C (pH 5.2). This thermal inactivation was irreversible. After an incubation of 5 min at 75 °C neither enzyme regained activity when further incubated for 1 h at 30 °C.

The loss of enzyme activity may be due to changes affecting the active site alone, which are not necessarily paralleled by a denaturation of the rest of the molecule. This effect can be directly determined by measuring changes in its intrinsic fluorescence. Both purified isoenzymes, Exgl and Exglb, yielded a single emission wave with a maximum centred at 344 nm when excited at 280 nm. After 30 min at 60 °C, a 78–80% absorption in the 344 nm band was observed for both enzymes. Again, the changes were irreversible since a further incubation of the denatured samples at 25 °C for 90 min showed almost no increase in the emission spectra (results not shown). As shown in Figure 6, no differences were observed in the thermal transition of Exgl and Exglb when determined by the disappearance of fluorescence. Both molecules exhibited half transition temperatures between 61.6 °C and 62 °C and a transition range of about 15 °C (59.5–74 °C). Similarly, denaturation kinetics followed by extinction of fluorescence did not show differences between Exgl and Exglb either. In both cases, after 40 min at 60 °C, the extinction of fluorescence was 65–70% of the initial value, and this decrease adjusted to first-order kinetics with a $t_{1/2}$ of 6 min. Accordingly, elongation of the second oligosaccharide of Exglb did not result in an increase in the thermal stability of the activity nor in a higher resistance of the molecule to thermal denaturation.

Finally, Exgl and Exglb were also equally susceptible to protease XIV from S. griseus or trypsin (results not shown).

**Figure 6** Thermal denaturation (unfolding) transition of Exgl (circles) and Exglb (triangles)

Samples of the purified enzymes in acetate buffer 25 mM, pH 5.2, supplemented with 20% glycerol, were subjected to progressive heating at a rate of 0.8 °C/min until 75 °C (solid symbols). Then, samples were cooled down at the same rate until 25 °C (open symbols). At the indicated temperatures, aliquots were taken to determine the intensity of the fluorescence. The concentration of the protein in the cuvette was 2 µg/ml.

**Figure 7** Hydropathic profile (Kyte and Doolittle, 1982) of the regions surrounding both glycosylation sites of Exgl.

(a) Site 1; (b) site 2.

**Conclusions on the frequency of elongation and endo H accessibility of Exgl oligosaccharides**

To our knowledge, the most complete study on glycosylation, endo H susceptibility and elongation of oligosaccharides has been carried out with yeast invertase (Trimble et al., 1983; Reddy et al., 1988; Ziegler et al., 1988). However, no conclusion could be reached from these studies in regard to oligosaccharide length and endo H susceptibility. However, a hydrophobic analysis has indicated that the most endo H-resistant oligosaccharides were located in the most hydrophobic regions of the molecules. Also, sequons with larger oligosaccharides tended to reside in more hydrophilic regions.

In the case of Exgl, the hydrophatic profiles (Kyte and Doolittle, 1982) of the sequences containing the glycosylation sites indicated that both sequons and their immediate neighbours were more hydrophilic than the surrounding sequons; however, no clear differences between sequons 1 and 2 were seen (Figure 7). However, quantification of the hydrophatic indices according to Eisenberg et al. (1984), using a window of seven residues centred at the glycosylated asparagine, indicated that, although both sequons are hydrophilic in nature, the first one ($H = -2.15$) was more hydrophilic than the second ($H = -0.69$). Finally, determination of Chou and Fasman (1978) conformational parameters indicated that both sequons were located in turns of the polypeptide chain (which are highly hydrophilic and exposed to the solvent) flanked by regions organized as α-helices or β-sheets (hydrophobic and probably more deeply positioned in the folded protein). Site 2, which was less hydrophilic than site 1, was also more resistant to endo H, a situation similar to that found in invertase. However, as far as elongation of the oligosaccharide is concerned, our results have followed the opposite pattern, i.e. the large oligosaccharide is on the less hydrophilic region.

We conclude that the hydrophobicity of a short stretch of seven to 10 residues does not influence significantly its frequency...
of elongation nor, probably, its accessibility to endo H. These parameters, which should be dependent on the tertiary structure of the folded protein, appear to be governed by different structural constraints, since the first oligosaccharide, which is never elongated, is more accessible to endo H, whereas the second one, which has some probability of being elongated, is more resistant to the endoglycosidase. It should be noted that the properties of the second oligosaccharide are reminiscent of those of oligosaccharide 11 from invertase, which is also long and partially resistant to endo H (Reddy et al., 1988; Ziegler et al., 1988).

Finally, with regard to secretion, we have previously believed that Exglb molecules bypass the Golgi cisterna in which the α-1,6-mannose backbone is added. However, the first oligosaccharide is never elongated even though the second one is, suggesting that all the molecules traverse that compartment, but each oligosaccharide has a different probability of being elongated (only 10% of the Exg molecules undergo elongation of the second oligosaccharides). High net negative charge and positioning of the oligosaccharide cores could be factors affecting their elongation frequency.

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