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), ExgIa, ExgIb 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]. ExgIb 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 ExgIa has at least one large oligosaccharide, since it has not been produced by mutant *mnn9*. To address the question of the origin of ExgIa, both sequons were individually mutated by substituting Gln for Asn. An ExgIa-like isoenzyme was still secreted by mutant Exg₁₆₅ but not by mutant Exg₃₂₅.

Additional studies on sequential deglycosylation of ExgIa 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 ExgIa contained two oligosaccharides, a short one bound to Asn¹⁶⁵ and a large one bound to Asn³²⁵, and, accordingly, originated from ExgIb. 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 ExgIb; 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 ExgII_{1/2}, here renamed as ExgIa, ExgIb and Exg₃₂₅. ExgIa and ExgIb 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 ExgII_{1/2}'), is also produced (Basco et al., 1993).

Figure 1 shows the primary structure of the several glycoforms, including that deduced for ExgIa in the present work. ExgIb 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 ExgI glycoforms

Wavy line, ExgI 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 ExgIa stems from the present study. The new and old nomenclature (the last one in parentheses) is indicated under each glycoform. Exg_{TM}, 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, phenylmethanesulphonyl fluoride.

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potential glycosylation sites (Asn¹⁶⁵ and Asn³²⁵) present in the polypeptide (Vázquez de Aldana et al., 1991; Basco et al., 1993). Exg₃₂₅ and Exg₁₆₅ carry a single oligosaccharide attached to the second and the first glycosylation sites respectively (Basco et al., 1993). ExgIa contains 30–40% carbohydrate, smears in SDS/acrylamide gels as other heavily glycosylated yeast glycoproteins (i.e. invertase, acid phosphatase), and its synthesis is prevented in mutant *mn9*; accordingly, it was suggested that this isoenzyme arose by elongation of the first, the second or both short oligosaccharides present in ExgIb (Ramírez et al., 1990). However, the more recent detection of Exg₃₂₅ and Exg₁₆₅ in the supernatant fluids of cell cultures (Basco et al., 1993) adds two new candidates to the possible precursors of ExgIa. 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 ExgIa arises from ExgIb by elongation of the oligosaccharide bound to the second glycosylation site (Asn³²⁵). The resulting enzyme, ExgIa, shows identical stability to thermal inactivation and resistance to proteases as its precursor (ExgIb). 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 (*MATa gal2*) and mutant CV55 (*MATa exg1 ura3-52 ino1-11 can'*), a strain in which the structural *EXG1* 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% bactopectone/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 *EXG1* 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 M13K07 helper phage (Messing, 1983).

Endo- β -N-acetylglucosaminidase (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 *EXG1* 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*, *SalI* and *XbaI* restriction sites into pRS316 vector (Sikorski and Hieter, 1989), since these sites are present in the *EXG1* gene. Site-specific mutants in the first, the second and both N-glycosylation sites of the *EXG1* gene were generated as previously described (Basco et al., 1993). Then, the resultant plasmids were digested with *SalI/XbaI* (site 1) or *KpnI/NaeI* (site 2) and fragments were subcloned in a plasmid (pRB1) obtained by inserting the *BamHI/ClaI* fragment of *EXG1* 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 Exg₃₂₅. For ion-exchange chromatography at high pressure (h.p.i.c.) dialysed samples (20–100 μ l) were applied to an ion-exchange (Bio-Gel TSK DEAE-5-PW) column (75 mm $\times$ 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 = 10⁵ 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 μ g 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 μ l). 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 μ l of 100 mM acetate buffer, pH 5.2, containing the following protease inhibitors: 2 mM phenylmethanesulphonyl fluoride (PMSF), 2 mM EDTA, 2 mM EGTA, 1 mM leupeptin, 1 mM pepstatin and 1 μ M 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 μ mol 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 (Morrissey, 1981). Standard deglycosylation reactions (5 m-units of endo H and about 15 units of exoglucanase in a final vol. of 280 μ l) 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 μ M).

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 *Exg*₁₆₅), the second (pRB3, mutant *Exg*₃₂₅) or both (pRB4, mutant *Exg*_{165,325}) 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 (*Exg*Ia) followed by a larger and sharper peak (*Exg*Ib). The major exoglucanases from site-directed mutants eluted as expected, i.e. *Exg*₃₂₅ (secreted by mutant *Exg*₁₆₅) eluted at a lower salt concentration than *Exg*₁₆₅ (secreted by mutant *Exg*₃₂₅), 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 *Exg*Ia-like enzyme was present in mutant *Exg*₁₆₅ (Figure 2c), no activity could be detected in this region of the eluent in samples from mutant *Exg*₃₂₅ (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

Figure 2 Exg glycoforms secreted by site-directed glycosylation mutants

Culture fluids from wild-type (a) and site-directed glycosylation mutants in the first (c) or the second (b) potential glycosylation sites, harvested in the middle exponential phase of growth, were concentrated and dialysed against 25 mM acetate buffer, pH 5.2, by ultrafiltration through Amicon membranes PM10 (exclusion size 10 kDa) and applied to a DEAE-Bio-Gel column (12 cm × 2.5 cm). Elution was performed as described in the Materials and methods section. (●), Exg activity; (---), NaCl gradient.

Figure 3 Time course of deglycosylation of ExgI

Purified *Exg*Ia (10 µg) (lane 2) was incubated with endo H under standard conditions. After 2 (lane 3), 3 (lane 4) and 24 (lane 5) h, samples were taken and analysed in an 8% acrylamide gel in the presence of SDS. Lane 1: deglycosylated (endo H) *Exg*Ib. Proteins were detected with the silver reagent.

that eluted with the enzyme produced in the presence of tunicamycin (results not shown).

Time course of deglycosylation of ExgI

Since the second oligosaccharide was only present in *Exg*₃₂₅, it seemed reasonable to consider this isoenzyme, in addition to *Exg*Ib, as a potential candidate for a precursor of *Exg*Ia. In order to answer this question, we subjected *Exg*Ia to a time-course deglycosylation with endo H and analysed the products by using SDS/PAGE. In the case of *Exg*Ib, 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 *Exg*Ia into the deglycosylated product. This result first suggested that *Exg*Ia had a single sugar residue and, accordingly, it originated from *Exg*₃₂₅. However, the same result would have been obtained if *Exg*Ia derived from *Exg*Ib, provided that only one *Exg*Ib 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 *Exg*Ia molecules.

We have reported preliminary evidence indicating that the first sugar residue of *Exg*Ib is more susceptible to endo H whereas the second masks 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 *Exg*₁₆₅ and *Exg*₃₂₅. As shown in Figure 4, *Exg*₃₂₅, which carries the oligosaccharide bound to the second sequon, was deglycosylated at a significantly lower rate than *Exg*₁₆₅. Assuming that elongation of the second residue did not alter significantly its susceptibility to endo H, we concluded that the approach

Figure 4 Time course deglycosylation of the major Exg secreted by glycosylation mutants

About 2 units of purified Exg₃₂₅ (a–d) and 1.3 units of purified Exg₁₆₅ (a'–d') were incubated with endo H (0.1 m-unit) in a final vol. of 25 μ l for 0 (a,a'), 2 (b,b'), 4 (c,c') and 8 (d,d') h. At these times, reaction mixtures were analysed on the TSK Bio-Gel column (h.p.l.c.). Symbols are as in Figure 2. For details of the elution times see Figure 5(c).

described in Figure 3 does not answer unequivocally the question of the origin of ExgIa.

Characterization of the *in vitro* deglycosylated ExgIa

In order to definitively answer the question of the origin of ExgIa, we analysed the deglycosylation products of ExgIa 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. ExgIa 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 ExgIa (a) from wild-type co-eluted with deglycosylated ExgIb one fraction before the deglycosylated ExgIa from mutant Exg₁₆₅ (b) which, in turn, co-eluted with the deglycosylated major Exg 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 ExgIa arose by elongation of the second oligosaccharide of ExgIb.

Figure 5 Analysis of the deglycosylation products of ExgI from (a) wild-type and (b) mutant Exg₁₆₅

The enzymes, purified as indicated in the text, were deglycosylated by treatment with endo H under standard conditions, and analysed on the TSK Bio-Gel column (h.p.l.c.) as described in the Materials and methods section. Arrows in (a) and (b) indicate the position of the deglycosylated major Exg secreted by the same strain. (c) Elution times (numbers) of the different forms of exoglucanases in the same column, as follows: a, wild type ExgIb; b, major Exg₃₂₅ (major peak from Figure 2c); c, Exg₁₆₅ (major peak from Figure 2b) and endo H-treated wild-type ExgIa and ExgIb; d, endo H-treated minor and major Exgs secreted by mutant Exg₁₆₅ (minor and major peaks from Figure 2c treated with endo H); e, endo H-treated Exg₁₆₅; f, Exg secreted in the presence of tunicamycin or Exg secreted by the double glycosylation mutant. Note that mutant Exg₁₆₅ secreted both Exg₃₂₅ and an ExgIa-like form and mutant Exg₃₂₅ secreted Exg₁₆₅.

Table 1 Comparison of the K_m values of purified Exgs I and II and their deglycosylated products *in vitro*

Enzyme	Endo H treatment	K_m	
		Laminarin (mg/ml)	p-NPG (mM)
ExgI	—	16.0 $\pm$ 2.0	4.2 $\pm$ 0.3
ExgI	+	8.1 $\pm$ 0.7	3.9 $\pm$ 0.3
ExgII	—	8.0 $\pm$ 0.5	3.9 $\pm$ 0.2
ExgII	+	7.9 $\pm$ 0.4	4.1 $\pm$ 0.2

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

We first compared the affinities of ExgIa, ExgIb and their endo H-deglycosylated products towards two different substrates, laminarin and p-NPG. As shown in Table 1, ExgIa exhibited half of the affinity for laminarin that ExgIb did, in spite of the fact

Figure 6 Thermal denaturation (unfolding) transition of ExgI (circles) and ExgII (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.

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 ExgIa, which might sterically hinder binding of the enzyme to substrates of high (laminarin) but not low (p-NPG) molecular mass. In contrast, the short sugar chains of both ExgIb 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, ExgIa and ExgIb, 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 ExgIa and ExgIb 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 ExgIa and ExgIb 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 ExgIb 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, ExgIa and ExgIb were also equally susceptible to protease XIV from *S. griseus* or trypsin (results not shown).

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

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

Conclusions on the frequency of elongation and endo H accessibility of Exg 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 Exg, the hydropathic 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 sequences; however, no clear differences between sequons 1 and 2 were seen (Figure 7). However, quantification of the hydropathic 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 ExgIb 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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