FEBS Letters 351 (1994) 155–158

FEBS 14438

The first hydrophobic segment of the ABC-transporter, Ste6, functions as a signal sequence

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Received 19 July 1994

Abstract

The Ste6 protein of *Saccharomyces cerevisiae* is a member of the ABC-transporter family containing 12 putative membrane spanning segments. To test whether Ste6 is inserted into the endoplasmic reticulum (ER) membrane by a sequential insertion mechanism we constructed a Ste6-invertase fusion containing the first hydrophobic segment of Ste6 fused to invertase lacking its own signal sequence. The resulting protein became glycosylated demonstrating that it was translocated across the ER-membrane. The finding that the N-terminal hydrophobic segment of Ste6 is recognized by the ER-translocation machinery suggests that Ste6 is inserted sequentially into the ER-membrane. Furthermore, our experiments support the N_{in} orientation of Ste6 predicted from the Ste6 sequence. Several findings suggest that invertase is cleaved from the Ste6 membrane anchor: (i) the gel mobility of deglycosylated wild-type invertase and fusion protein derived invertase is the same; (ii) the periplasmic invertase activity is found in the cell wall fraction, i.e. it is not associated with the cell body; (iii) a signal peptide cleavage site is predicted in the Ste6 sequence. Although the membrane anchor appeared to be cleaved, most of the invertase was retained in the ER, probably due to aggregate formation.

Key words: Secretion; Mating; Endoplasmic reticulum; Translocation; Glycosylation

1. Introduction

The Ste6 protein of *Saccharomyces cerevisiae*, which is required for the secretion of the mating pheromone a-factor, contains two copies of a conserved ABC-transporter motif consisting of six putative membrane spanning segments and an ATP-binding domain [1,2]. We were interested in how the Ste6 protein is inserted into the membrane. It is assumed that most eukaryotic transmembrane proteins are sequentially inserted into the membrane by the ER-translocation machinery [3]. Hydrophobic sequences are crucial for attaining the proper membrane topology, acting alternately as signal-anchor and stop-transfer sequences [4,5]. The context around the first N-terminal hydrophobic segment seems to determine the general orientation of the protein in the membrane (N_{in} or N_{out}) [6].

However, there are also transmembrane proteins which seem to spontaneously insert into a membrane [7]. Possible examples are eukarotic proteins with a single C-terminal hydrophobic segment like cytochrome b_5 [8] or bacterial toxins like the colicins [9]. The α-subunit of the SRP receptor also seems to insert into the membrane by an unusual mechanism [10]. To decide whether Ste6 is inserted into the membrane by a sequential insertion mechanism via the ER translocation machinery or by another mechanism we fused the first N-terminal hydrophobic sequence, which is preceded by a 25 amino acid N-terminal extension, to the secretory protein invertase as reporter protein. We could show that the first putative transmembrane segment of Ste6 functions as a signal sequence directing invertase into the secretory pathway. This indicates that Ste6 is inserted into the ER membrane by the translocation machinery. Our results further confirm the prediction about the general orientation of Ste6 in the membrane (N_{in}) deduced from the protein sequence.

2. Materials and methods

2.1. Plasmid construction

pRK172 is based on the 2μ-vector YEp420 [11]. It contains: a 850 bp *SphI/SalI PDC1* promoter fragment [12], a 352 bp *STE6* PCR fragment (pos. 451–702) [1,2] with attached *Bam*HI and *Sal*I sites and a 2.1 kb *Sal*I invertase fragment from plasmid pINVSal [13]. The amino acid sequence at the junction of the two fragments of the Ste6-invertase fusion is:

...Ste6..SMAV(78) ↓ VD T(25)SDR ...Suc2....

2.2. Enzyme assays

Cultures were grown in medium containing 2% glucose. The expression of the secreted form of invertase was induced by growth in 0.1% glucose medium for 3 h. Cells were harvested and washed in 10 mM NaN_3, 10 mM NaF. For spheroplasting cells were resuspended in 1.0 M sorbitol, 50 mM HEPES, 10 mM NaN_3, pH 7.5. After 20 min pretreatment with 10 mM DTT, cells were spheroplasted with oxalyticase (Enzogenetics) at about 1 μg (35 U) per A_{600} unit of cells for 30 min at 37°C. For cell extract preparation cells were resuspended in 0.3 M sorbitol, 50 mM HEPES, 10 mM NaN_3, pH 7.5 and disrupted with glassbeads. Invertase activity was assayed according to Goldstein and Lampen [14]. Glucose-6-phosphate dehydrogenase activity was determined according to the following protocol. The sample in 800 μl 50 mM Tris-Cl, pH 7.5, was mixed with 100 μl of 5 mM $NADP^+$ (in 1% $NaHCO_3$) and preincubated for 5 min at room temperature. Then 100 μl of 6.6 mM glucose-6-phosphate were added and the rate of increase in absorbance at 340 nm was registered in a spectrophotometer. Strains are listed in Table 1.

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SSDI 0014-5793(94)00825-6

Table 1
Yeast strains

Strain	Genotype	Source
DBY2063	*MATa leu2-3,112 ura3-52*	David Botstein (Stanford)
PS42-1A	*MATa his3 leu2 lys2 pep4::HIS3 suc⁰ trp1 ura3*	Hans Dieter Schmidt (Göttingen)
HMSF1	*MATa sec1-1*	Yeast Genetic Stock Center
SF294-2B	*MATa sec7-1*	Yeast Genetic Stock Center
HMSF190	*MATa sec23-1*	Yeast Genetic Stock Center

3. Results and discussion

To generate the *STE6-SUC2* gene fusion a *STE6* PCR fragment coding for the N-terminal part of Ste6 (amino acids 1 to 78) including the first hydrophobic segment, was ligated to part of the *SUC2* gene encoding the invertase sequences downstream from the signal peptidase cleavage site (Ala-19 ↓ Ser-20) lacking the hydrophobic signal sequence [15,16]. The gene fusion was expressed in the yeast *suc⁰* strain PS42-1A under the control of the *PDC1* promoter from a multi-copy plasmid. As can be seen from Fig. 1, lane 13, protein bands in the range of about 80–100 kDa could be detected on Western blots with anti-invertase antibodies. The apparent size of the fusion protein (67 kDa) was larger than calculated from the protein sequence. However, upon treatment of the cell extracts with endoglycosidase F (Endo F), an enzyme which removes N-linked sugar chains, the mobility on the gel was increased (Fig. 1, lane 14) demonstrating that the invertase portion of the fusion protein was glycosylated and therefore had access to the lumen of the ER. The glycosylation pattern resembled core-glycosylated invertase lacking the outer-chain glycosylation. We therefore compared the glycosylation pattern of the protein derived from the *STE6-SUC2* fusion with wild-type invertase in secretory mutants blocked at different stages of the secretory pathway [17,18]. At non-permissive temperature (37°C) the *sec1-1* mutant with a late block in secretion, i.e. post-Golgi (Fig. 1, lane 3), and the *sec7-1* mutant with an intra-Golgi block in secretion (Fig. 1,

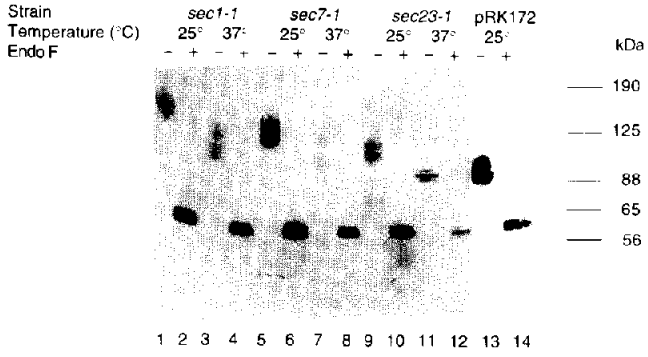

Fig. 1. Western blot with anti-invertase antibodies. The glycosylation pattern of invertase induced in *sec* mutant strains grown at permissive (25°C) or non-permissive temperature (37°C) was compared to the pattern observed with *suc⁰* strain PS42-1A transformed with plasmid pRK172 containing the *STE6-SUC2* fusion. 1,2: *sec1-1* (25°C); 3,4: *sec1-1* (37°C); 5,6: *sec7-1* (25°C); 7,8: *sec7-1* (37°C); 9,10: *sec23-1* (25°C); 11,12: *sec23-1* (37°C); 13,14: *suc⁰* + pRK172. Even numbers, samples treated with Endo F: odd numbers, samples not treated with Endo F. Lanes 13 and 14 were exposed for a shorter time.

lane 7) gave rise to a diffuse staining pattern on Western blots with anti-invertase antibodies characteristic of fully or partially outer-chain glycosylated invertase. The glycosylation pattern of invertase observed in a *sec23-1* mutant with an early block in secretion between ER and Golgi (Fig. 1, lane 11) closely resembled the pattern observed with the Ste6-invertase fusion (Fig. 1, lane 13). We therefore conclude that our fusion construct is mainly localized in a pre-Golgi compartment, most probably the ER.

The Ste6-invertase fusion as calculated from the protein sequence is 8.8 kDa larger than wild-type invertase, a difference which should be easily discernible on a 7.5% SDS-PAGE gel. Unexpectedly, however, the deglycosylated proteins both migrated with the same mobility on SDS gels (Fig. 1). We therefore conclude that the N-terminal Ste6 sequences were removed by signal peptidase upon transit into the lumen of the ER. In agreement with this interpretation a potential signal cleavage site is predicted within the Ste6 sequence (Gly62 ↓ Ser63; Fig. 2) according to the rules set up by von Heijne [19].

If the Ste6 membrane anchor is cleaved, soluble secreted invertase should be detectable outside of the cell. Indeed, we could show that in the case of the Ste6-inver-

Table 2
Periplasmic invertase activity

	Invertase μg glucose			Glucose-6-phosphate dehydrogenase nMol NADPH	
	A_{600} · min · ml			A_{600} · min · ml	
Strain	Periplasm	Total	% Periplasm	Periplasm	Total
DBY2063 (2%)	0	40	0	0	14
DBY2063 (0.1%)	300	410	73	0	24
suc⁰/pRK172	106	430	25	0	16

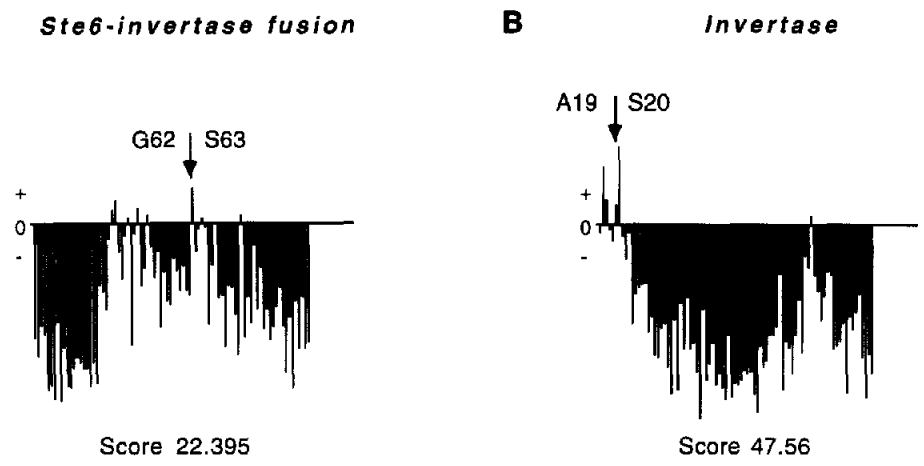

Fig. 2. Signal peptide cleavage site prediction. The program PLOT.A/SIG which is part of the MacPROT package of Lüttge and Markiewicz was used to predict the potential signal peptide cleavage site for (A) the Ste6-invertase fusion protein encoded by pRK172 and for (B) wild-type invertase. The potential cleavage sites with the highest probability are shown.

tase fusion about 25% of the cell associated invertase activity was found in the periplasm compared to 75% in the case of wild-type invertase (Table 2). This activity cannot be attributed to cell lysis since no activity could be detected for the cytoplasmic enzyme glucose-6-phosphate dehydrogenase under these conditions. In order to see whether the invertase activity was plasma membrane-associated the cell wall was removed with oxalyticase and a soluble cell wall fraction was separated from the spheroplasts by low speed centrifugation. After removal of the cell wall the periplasmic invertase activity was found in the soluble fraction demonstrating that the protein derived from the *STE6-SUC2* fusion is not anchored to the plasma membrane (Table 3). Only about 10% of the glucose-6-phosphate dehydrogenase activity is released into the soluble fraction under these conditions indicating that the spheroplasts remained mostly intact during the experiment.

Virtually all of the fusion protein appears to be cleaved. Why then is such a large fraction of the invertase retained in the ER? In order to test whether this retention was due to aggregate formation cell extracts were prepared and spun at 100,000 × *g* for 1 h. The supernatant and pellet fractions were analyzed by SDS-PAGE and Western blotting with anti-invertase antibodies (Fig. 3). Two distinct bands labeled I and II in Fig. 3 and a diffuse protein smear on top of the two bands could be distinguished. Band I and the diffuse protein species were predominantly found in the supernatant fraction (Fig. 3, lane 2) while band II was mostly found in the pellet fraction (Fig. 3, lane 3). After treatment of the cell extract with the detergent Triton X-100, which should release membrane-enclosed invertase, essentially the same distribution was observed although a higher fraction of total invertase protein was found in the soluble fraction (Fig. 3, lane 5). The band II protein was still exclusively present in the pellet fraction (Fig. 3, lane 6). This form, which constitutes about half of the total invertase protein, therefore seems to correspond to an insoluble form of invertase. How the band I and band II proteins differ from each other is not clear. The proteins could be differently processed or glycosylated forms of invertase.

4. Conclusion

In this report we show that the first hydrophobic segment of Ste6 functions as a signal sequence suggesting that Ste6, as proposed for other transmembrane proteins, is sequentially inserted into the ER-membrane by the translocation machinery. The first transmembrane segment of a multispanning membrane protein determines the orientation of the protein in the membrane [6]. The finding that invertase attached to the first hydrophobic segment of Ste6 is directed into the ER suggests an N_{in} orientation for Ste6. This is in line with the topological model derived from the protein sequence [1,2]. The Ste6 membrane anchor appears to be cleaved from the

Table 3
Fractionation of periplasmic invertase activity: *suc*$^{-}$/pRK172 strain

	Invertase μg glucose	Glucose-6-phosphate dehydrogenase nmol NADPH
	A_{600} · min · ml	A_{600} · min · ml
Intact cells	140	–
Cell wall fraction	154	2.6
Spheroplasts	490	20
% in cell wall fraction	24	13

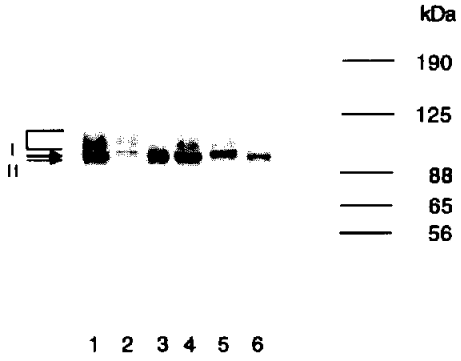

Fig. 3. Fractionation of invertase protein. Cell extracts of the *suc*^{0} strain PS42-1A transformed with pRK172 were fractionated by centrifugation into supernatant (2,5) and pellet fraction (3,6). 1,4: total cell extract. Lanes 1–3, extract not treated with Triton X-100; lanes 4–6, extract treated with 1% Triton X-100 for 10 min at 37°C. Equal portions of the fractions were loaded onto the gel.

fusion protein. Whether this also occurs in the context of the normal Ste6 protein remains to be shown. A large portion of the cleaved invertase is retained in the ER probably in the form of protein aggregates. Why does the invertase derived by cleavage from the Ste6-invertase fusion behave differently from wild-type invertase? The explanation could be that a close coordination between signal peptide cleavage, glycosylation and folding is required to achieve proper folding and oligomerization of invertase. If signal peptide cleavage occurs at a slower rate due to the presence of an unnatural cleavage site this could lead to an aberrant folding of the protein and aggregate formation.

Acknowledgements: We would like to thank Hans Dieter Schmidt for sending us the *suc*^{0} strain. This work was supported by the BMFT project: 'Stoffumwandlung mit Biokatalysatoren' and by the DFG grant to R.K. (Ko 963/2-1).

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