The nascent polypeptide-associated complex (NAC) of yeast functions in the targeting process of ribosomes to the ER membrane

Brigitte Wiedmann*, Siegfried Prehn

Department of Biochemistry, Humboldt University, Charité, Hessische Str. 3-4, 10115 Berlin, Germany

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Abstract We study here the binding of ribosomes to the endoplasmic reticulum (ER) membrane and its dependence on nascent polypeptide-associated complex (NAC). For this, we use an in vitro translation system in combination with isolated microsomes. Importantly, all components in the system are derived from a single source, *Saccharomyces cerevisiae*. Ribosome nascent chains (RNCs) of the two naturally occurring invertase species (secreted or cytosolic) were prepared in wild-type, ΔαNAC or $\Delta\alpha\beta_1\beta_3$NAC translation lysates and tested for binding to the corresponding microsomal membranes. We provide evidence that NAC prevents binding of RNCs without a signal sequence to yeast membranes. In the absence of NAC, signal-less RNCs are able to bind to ER membranes. However, following puromycin treatment, only very few nascent chains translocate into the lumen, as detected by glycosylation.

Key words: Nascent polypeptide-associated complex; Ribosome targeting; Flotation assay; Yeast

1. Introduction

The nascent polypeptide-associated complex (NAC) has puzzled quite a number of laboratories for the past 5 years. Originally discovered as proteins participating in transcription [1–4], the subunits of NAC were shown to play a role in protein targeting to the endoplasmic reticulum (ER) membrane in our laboratory [5–8]. Using similar but not identical assay systems, other laboratories questioned this function of NAC [9,10]. All the in vitro assay systems were based on components of extremely diverse origins, e.g. wheat germ or rabbit reticulocyte translation components, membranes from dog pancreas and mRNA encoding bovine, firefly, bacterial or yeast proteins. NAC had to be 'washed off' in order to show the system's dependency upon it. The microsomal membranes differed due to the different methods used to remove the ribosomes. Therefore, the completeness of the removal of NAC from ribosome nascent polypeptide complexes (RNCs), the purity of the membranes and especially the amount of NAC that could be applied back to the system were accounted to be the main reasons for the different results [11].

Flotation assays based only on yeast components were developed because they seemed to be the best system for examining the role of NAC in protein targeting. We also wanted to see whether NAC has the same function in yeast, even though the three subunits are not essential in yeast in contrast to mice [12] and *Drosophila* [13]. We show here that NAC prevents the binding of RNCs without signal sequence to the microsomal membranes. Binding occurred in the absence of NAC but only a few 'wrong' proteins were translocated and glycosylated, which underlines the finding of Jungnickel and Rapoport [14] that the signal sequence possesses a second function, namely, the ability to open the translocation pore.

2. Materials and methods

2.1. Strains

Wild-type (WT) *Saccharomyces cerevisiae* W303 (*MATα ade2 his3-11,15 leu2-3,113 trp1-1 ura3-1*). The following knockout mutants of *S. cerevisiae* strain W303 were constructed according to Schneider et al. [15]: Δα = W303 *egd2*$^-$ and $\Delta\alpha\beta_1\beta_3$ = W303 *egd*$^-$, *egd1*$^-$, *btt1*$^-$.

2.2. Preparation of in vitro translation lysates

Lysates were prepared from 10 l log phase cultures as described [16] with the exception that the separation of cytosol and microsomes occurred at 40 000 × *g*. The supernatant was applied to a Sephadex G25 column and fractions of the highest optical density at 260 nm (OD_{260}) were pooled and frozen as droplets in liquid nitrogen. The OD_{260} values of the lysates prepared from the WT, the Δα and the $\Delta\alpha\beta_1\beta_3$ strains were 131, 138 and 96, respectively. The OD_{260} to OD_{280} ratio of all three lysates was 1.6.

2.3. Preparation of RNC

Ribosomes carrying the N-terminal 97 amino acids of WT invertase (WT-Inv.), 77 amino acids of invertase lacking the signal sequence (ΔSS-Inv.) or 72 amino acids of chloramphenicol acetyl transferase (CAT) were created by the truncated messenger method [17]. Essentially, plasmids containing the genes of the mentioned peptides were linearized with appropriate restriction enzymes (*Hin*fI for invertase, *Eco*RI for CAT) and transcribed with SP6 polymerase. Since the mRNAs lacked the stop codon, translated peptides remain bound to ribosomes and can therefore be isolated by sedimentation. Yeast lysates were treated with S7 nuclease prior to programming with the exogenous mRNAs. 100 μl translation assays were sedimented through a low salt cushion (30 min, 100 000 × *g*, Beckman, TL 120.2 rotor, ribosome binding buffer (RBB) with 500 mM saccharose: 50 mM HEPES, pH 7.4, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, protease inhibitor mix) and resuspended in 50 ml RBB.

2.4. Preparation of microsomal membranes

The 40 000 × *g* pellet of the translation lysate preparation served as origin of EDTA and high salt-washed rough membranes (EKRM). Membranes were resuspended in 50 mM HEPES, pH 7.4, 250 mM sucrose, 500 mM KOAc, protease inhibitor mix and kept on ice for 15 min. The membranes were sedimented by a 30 min centrifugation at 40 000 × *g* through two volumes of a high salt cushion (50 mM HEPES, pH 7.4, 500 mM sucrose, 500 mM KOAc, protease inhibitor mix) in a Beckman TLA 100.4 rotor. They were resuspended in low salt buffer (50 mM HEPES, pH 7.4, 150 mM KOAc, protease inhibitor mix) with 50 mM EDTA and incubated on ice for 15 min. The membranes were sedimented through a high salt cushion, as described above, after adjustment of the KOAc concentration to 650 mM and

*Corresponding author. Fax: (49) (30) 2093 7217.
E-mail: brigitte.wiedmann@charite.de

Abbreviations: CAT, chloramphenicol acetyl transferase; ΔSS, without a signal sequence; ER, endoplasmic reticulum; NAC, nascent polypeptide-associated complex; RNC, ribosome nascent polypeptide complex; WT, wild-type

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incubation on ice for a further 10 min. Membranes were resuspended in high salt buffer and sedimented a third time through a high salt cushion followed by two low salt washing steps. The final sediments were resuspended in low salt buffer and adjusted to an equal OD ($OD_{260} = 45$).

2.5. Flotation assay

10 μl EKRM with 2, 4 or 8 μl RNCs were adjusted to 20 μl with RBB and incubated for 5 min at 28°C and 15 min on ice. This 20 μl was thoroughly mixed with 200 μl of 2.3 M sucrose in RBB, poured into centrifuge tubes (Beckman, Ultra-Clear 5×41 mm) and overlaid with 360 μl 1.9 M sucrose in RBB. The tubes were filled up with RBB and centrifuged for 3.75 h at 40 000 rpm in a Beckman Ti 51.1 rotor with adapters. The tubes were then frozen in liquid nitrogen and cut into a 300 μl bottom fraction (B) and a 400 μl top fraction (T). Sediments were solubilized with 1% sodium dodecyl sulfate (SDS). Fractions were diluted with 600 μl (B) or 500 μl (T) water, 50 mg tRNA was added and the proteins/peptides were precipitated with TCA. The sediment was solubilized in 50 μl alkaline sample buffer (pH 8.8) and submitted to SDS-polyacrylamide gel electrophoresis (PAGE) followed by fluorography or Western blotting.

2.6. Glycosylation of invertase in vitro

4 μl RNCs of WT or ΔSS-Inv. was incubated with 10 μl EKRM of the corresponding strain for 5 min at 28°C and 15 min on ice. Puromycin (final concentration 1 mM) and RNAse ONE (Promega) were added and the mixture was incubated at 30°C for 10 min. Proteins were separated on 16% SDS-polyacrylamide gels after TCA precipitation and visualized by fluorography.

2.7. Proteinase K protection

RNCs were targeted to the membranes as described above. The sample was divided into four aliquots and three of them were treated with puromycin and RNAse ONE. Two aliquots then received proteinase K (final concentration 1 mg/ml), one after addition of Triton X-100 (1% final concentration). The reaction took place on ice for 1 h and was stopped by addition of the same volume of Laemmli sample buffer.

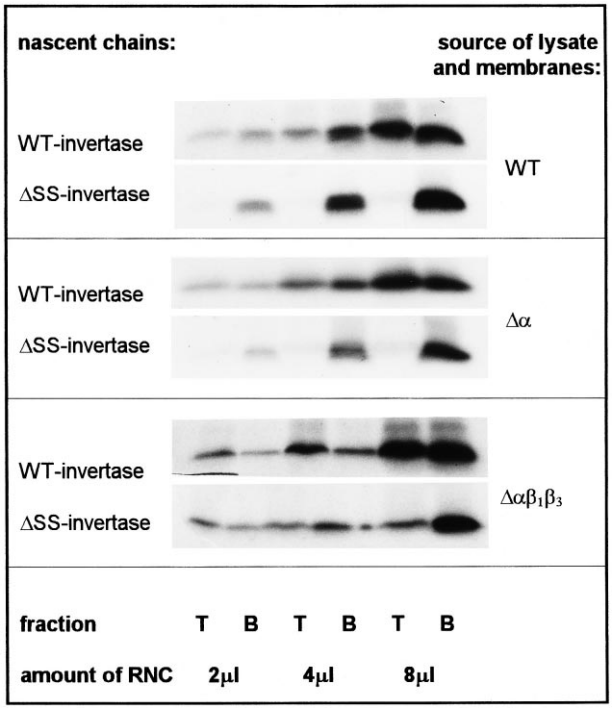

Fig. 1. NAC prevents the targeting of RNCs of cytosolic proteins to the ER membrane. [^{35}S]methionine-labelled RNCs of WT and ΔSS-Inv. were produced by in vitro translation in lysates of the indicated yeast strains (right hand panel). Different amounts (2, 4 and 8 μl) of RNCs were incubated with a constant amount of the corresponding membranes. This mixture was separated by sucrose gradient centrifugation into a top fraction (T) containing floated membranes with attached RNCs and the bottom fraction (B) with sedimented RNCs. Proteins of the fractions were separated by SDS-PAGE and visualized by fluorography.

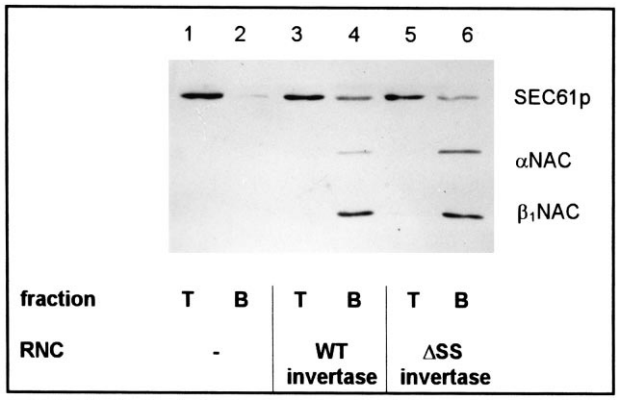

Fig. 2. Ribosomes with associated NAC are not able to bind to ER membranes. Membranes of WT cells were floated directly or after incubation with 4μl RNCs of WT or ΔSS-Inv., respectively, as described in Fig. 1. The distribution of *SEC61p* (membrane marker protein) and the NAC subunits (ribosome marker proteins) was analyzed by Western blotting.

2.8. Glycosylation of invertase in vivo

Plasmids encoding WT or ΔSS-Inv. [18] were transformed into $\Delta\alpha\beta_1\beta_3$ yeast cells. Expression of the plasmid was induced by changing the growth medium from 2% glucose to 1% galactose/1% sucrose. 10 ml of log phase cells were lysed in IP buffer (15 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1% SDS after 5 h labelling with [^{35}S]methionine. The cell-free supernatant was obtained by centrifugation and 10-fold diluted with IP buffer without SDS. Incubation with invertase antibodies occurred overnight on a roller at 4°C. IgGs were collected by addition of protein A-Sepharose. The proteins were eluted with Laemmli sample buffer after five washing steps, separated on 10% SDS polyacrylamide gels and visualized by fluorography.

3. Results

Translation lysates with a comparable activity were prepared from three different yeast strains: from WT, Δα and $\Delta\alpha\beta_1\beta_3$ knockout cells. The 40 000×*g* pellets obtained in the course of these preparations were purified from ribosomes by several washes with high salt buffer and EDTA to yield EKRMs. These EKRMs floated almost completely in a sucrose gradient (judged by immunodetection of the *SEC61* protein, Fig. 2, first two lanes, and by Coomassie staining, data not shown). NAC proteins were removed completely, the ribosomal protein L3 was left in traces only. We chose invertase either with or without its signal sequence and CAT as model peptides. The transcripts of the corresponding linearized plasmids translated equally well in all three lysates. RNCs were isolated by centrifugation through a low salt sucrose cushion.

The positive control, RNCs of the signal sequence bearing invertase, was targeted to the membranes and hence floated with them regardless of the translation lysate and membranes used. RNCs of signal-less invertase sedimented when produced in NAC containing lysate. In contrast, they were able to bind to membranes and floated with them when the whole NAC was deleted. The deletion of the α subunit alone did not affect targeting of signal-less invertase (Fig. 1). The β subunit

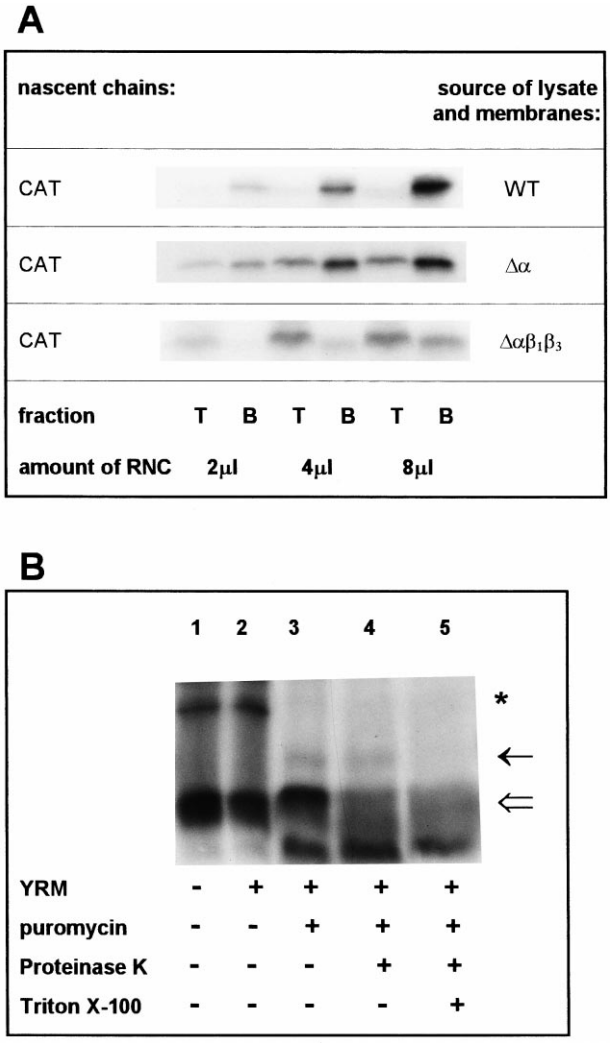

Fig. 3. Deletion of NAC causes targeting of CAT to the ER membrane. A: An experiment as described in Fig. 1 was performed but RNCs of CAT were used instead of invertase. B: The higher molecular weight band (marked by ←) that appeared after release of CAT nascent chains (marked by ⇐) from ribosomes by puromycin in a Δα lysate was protected from proteinase K. An asterisk marks the complex of CAT and tRNA which disintegrated by puromycin treatment (lanes 3–5).

prevented the binding of these RNCs to membranes of the α knockout strain comparable to the whole NAC of WT RNCs to the corresponding membranes.

The influence of NAC on the targeting of ribosomes to the membrane also became evident because it was detected exclusively in the bottom fraction of a flotation assay when we added RNCs (Fig. 2, lanes 4 and 6), whereas the majority of *SEC61p* was located in the top fraction (Fig. 2, lanes 1, 3 and 5). With the addition of RNCs, the *SEC61p* signal became slightly stronger in the bottom fractions (lanes 4 and 6). We always detect a trace of *SEC61p* (e.g. microsomal remnants) in the ribosomal pellet of our translation lysates (data not shown). These microsomes do not float. NAC is brought into our assay system with the ribosomes since the trace of microsomes can not carry such an amount of NAC. Also, NAC binds to ribosomes but not to membranes (data obtained from cell fractionation experiments, crosslink experiments to nascent peptides [18], binding of recombinant NAC to ribosomes, our unpublished results). We therefore conclude that NAC containing ribosomes (non-translating or those with nascent chains of non-secretory proteins in case of WT yeast) sediment because NAC abolishes the binding to the membrane.

CAT was chosen as a second signal-less model peptide. This cytoplasmic protein of bacterial origin was not targeted at all, when NAC was present (WT, B fractions, Fig. 3A). Deletion of α NAC caused a small fraction of the RNCs to float (Δα NAC, T fractions, Fig. 3A). Removal of the whole complex increased the amount of membrane-bound RNCs ($\Delta\alpha\beta_1\beta_3$ NAC, T fractions, Fig. 3A).

Next, we tested whether binding of RNCs to membrane vesicles automatically leads to translocation of the nascent chains into the lumen. We exploited the existence of potential glycosylation sites in all three peptides. WT invertase peptides became glycosylated upon release of the nascent chains from bound RNCs with puromycin and RNAse (Fig. 4A, band marked by →). A faint higher molecular signal was observed for signal-less invertase, but independent of NAC (Fig. 4A, band somewhat lower then the one marked by →). These peptides were proven to be glycosylation products, since they bound to CON A-Sepharose (Fig. 4B). Even a small amount of the CAT peptide became glycosylated. This was shown by endoglycosidase H sensitivity of the higher molecular band, binding to CON A-Sepharose (data not shown) and by resistance to proteinase K digestion (Fig. 3B, band marked by →).

The result obtained in vitro was then tested by an in vivo labelling experiment. The triple knockout strain was transformed with galactose inducible plasmids encoding either

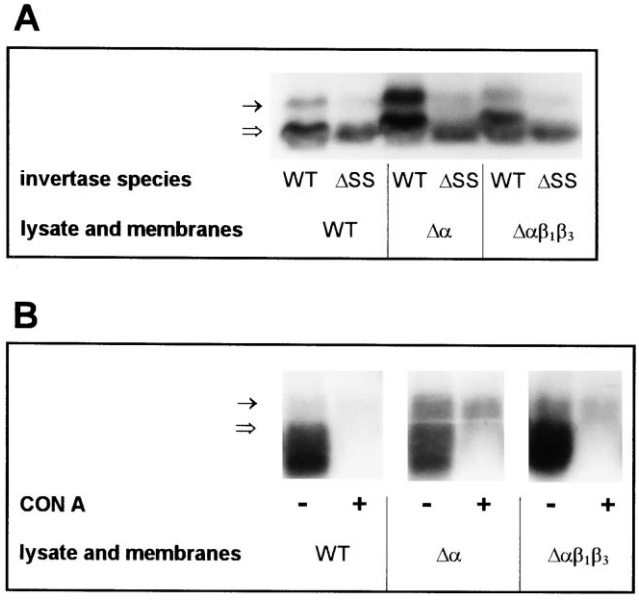

Fig. 4. Fractions of targeted WT and ΔSS-Inv. peptides were translocated into the ER lumen as shown by the appearance of a glycosylated product. RNCs of WT and ΔSS-Inv. were produced in lysates of the three indicated strains and incubated with the corresponding membranes as done in previous experiments. A: A higher molecular weight band (marked by →) appeared when the nascent chains (marked by ⇒) were released from the ribosome by treatment with puromycin and RNAse. The smaller nascent peptide of ΔSS-Inv. runs slightly below the WT invertase peptide. B: The higher molecular weight product of ΔSS-Inv. peptides (marked by →) bound to CON A-Sepharose.

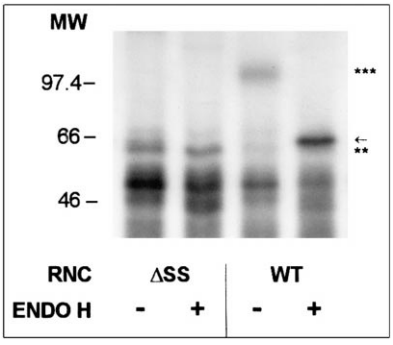

Fig. 5. WT invertase but not ΔSS-Inv. becomes glycosylated in vivo in a NAC deletion mutant. The $\Delta\alpha\beta_1\beta_3$ mutant was transformed with plasmids encoding WT or ΔSS-Inv. and labelled with [^{35}S]methionine for 5 h. Cells were lysed, invertase-immunoprecipitated and one half of each sample was treated with endoglycosidase H. *** = secreted glycosylated invertase, ⇐ = product of endoglycosidase H treatment, * = cytosolic invertase. The smaller proteins present in all four lanes are likely degradation products.

WT invertase or ΔSS-Inv. Expression of the plasmids was induced by changing the substrate from glucose to 1% galactose/1% raffinose in the presence of [^{35}S]methionine for 5 h. Invertase species were immunoprecipitated and one half of the sample was treated with endoglycosidase H (Fig. 5). This converted the broad band in the WT lane (***) to a 63 kDa protein (*). In strains transformed with the plasmid encoding ΔSS-Inv., the cytosolic invertase species (←) was immunoprecipitated. There was no glycosylated invertase species detectable and endoglycosidase H treatment did not change the protein pattern of ΔSS-Inv. In all samples, smaller proteins were co-precipitated. These are most likely degradation products.

4. Discussion

We demonstrate here that yeast NAC functions in the targeting process of RNCs to the ER membrane. It prevents the binding of ribosomes translating proteins which do not follow the secretory pathway. This function is supported by NAC's location. We found it almost exclusively bound to ribosomes [18] and failed to detect any NAC in the nucleus, whatever method we applied. The results described herein equal those obtained in the mammalian system [5,7,8,11]. We assume that controverse results as published by [9,10] can be attributed to methodological aspects.

Yeast cells do not express a growth defect when one, two or even all three subunits of NAC are deleted [18]. We have shown previously that there is no (or not detectable) missecretion of invertase lacking a signal sequence in vivo [18]. Another proofreading step had to be assumed following targeting of RNCs to the membrane, because in vitro experiments demonstrated binding of RNCs of cytosolic proteins in NAC mutants, which could occur during translocation or afterwards by degradation. Two experiments were performed to address this question. First, glycosylation and/or protection from proteinase K digestion was examined as proof for translocation. We detected in vitro only a minor translocation of non-secretory peptides that were targeted to the ER membrane. Second, we transformed NAC mutants with plasmids encoding either secretory or cytosolic invertase and immunoprecipitated invertase after metabolic labelling with [^{35}S]methionine. We did not detect glycosylated invertase in vivo. The steady state concentration of the cytoplasmic invertase species was always lower then that of the secreted form. We tested several independent clones and WT yeast, always with the same result. We assume that this invertase species became degraded while it was still in the cytosol. Both experiments support the finding of Jungnickel at al. [14] that the cell developed a second proofreading step for proteins that follow the secretory pathway. The signal sequence of secretory proteins is necessary to open the translocation pore. Obviously, even for such a low eukaryotic cell as *Saccharomyces*, it is an advantage to possess several cytosolic (SRP, NAC, heat shock proteins, etc.), membrane (docking protein, *SEC61p*, etc.) and lumenal (*KAR2*p, etc.) proteins which control that intracellular protein transport is correct and efficient.

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