L25 functions as a conserved ribosomal docking site shared by nascent chain-associated complex and signal-recognition particle

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The nascent chain-associated complex (NAC) is a dimeric protein complex of archaea and eukarya that interacts with ribosomes and translating polypeptide chains. We show that, in yeast, NAC and the signal-recognition particle (SRP) share the universally conserved ribosomal protein L25 as a docking site, which is in close proximity to the ribosomal exit tunnel. The amino-terminal segment of β -NAC was found to be required for L25 binding. Purified NAC can prevent protein aggregation *in vitro* and thus shows certain properties of a molecular chaperone. Interestingly, the α -subunit of NAC interacts with the 54 kDa subunit of SRP. Consistent with a regulatory role of NAC in protein translocation into the endoplasmic reticulum (ER), we find that deletion of NAC results in an induction of the ER stress-response pathway. These results identify L25 as a conserved interaction platform for specific cytosolic factors that guide nascent polypeptides to their proper cellular destination.

Keywords: molecular chaperone; L25; NAC; ribosomes; SRP

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INTRODUCTION

The process of *de novo* protein synthesis requires the coordinated activity of many cytosolic factors in addition to the components that are directly involved in transcription and translation. When emerging from the ribosome, newly synthesized polypeptide chains are immediately bound by molecular chaperones that assist in subsequent folding, or by specific targeting factors that help nascent chains to reach their proper cellular topology (Bukau *et al*, 2000; Hartl & Hayer-Hartl, 2002). For example, signal sequences of secretory proteins are co-translationally recognized by the signal-recognition particle (SRP), which targets these nascent chains to the endoplasmic reticulum (ER; Keenan *et al*, 2001).

In the eukaryotic cytosol, one of the first components contacting a nascent polypeptide is the nascent chain-associated complex (NAC)—a highly conserved heterodimeric protein that interacts with ribosomes (Wiedmann *et al*, 1994). According to the recently solved crystal structure of the archaeal protein, NAC subunits contain unique, so-called NAC domains for dimerization and a carboxy-terminal UBA domain (ubiquitin-associated domain), which is not present in eukaryotic β -NAC (Spreter *et al*, 2005; Fig 1A). Whereas its binding site on the ribosome, as well as its exact function, has remained elusive, NAC can be crosslinked to short nascent chains, preferably of non-secretory proteins, and has been shown to dissociate from target polypeptides following chain release from the ribosome (Wang *et al*, 1995). A regulatory role for NAC in protein translocation into the ER was proposed (Lauring *et al*, 1995). Some of these properties are reminiscent of those of trigger factor (TF), a structurally unrelated protein that functions as the most prominent nascent chain-binding chaperone in bacteria (Valent *et al*, 1995; Hestekamp *et al*, 1996). Both TF and bacterial SRP dock specifically onto L23 of the large ribosomal subunit, in close proximity to the ribosomal exit tunnel (Kramer *et al*, 2002; Gu *et al*, 2003).

Here, we provide evidence that, in eukaryotes, NAC binds to the ribosomal protein L25, the homologue of L23 and a known contact site for eukaryotic SRP (Pool *et al*, 2002). Thus, L23/25 functions as a conserved binding platform for cytosolic factors interacting with nascent chains, although TF is restricted to bacteria and NAC to archaea and eukarya. Moreover, we present evidence that NAC interacts directly with SRP, in support of the view that both components have closely related functions in protein biogenesis.

RESULTS

NAC α - and β -subunits are functionally conserved

NAC is generally found in eukarya and archaea but not in bacteria. Eukaryotic NAC contains two homologous subunits, designated α and β . The yeast genome encodes one α -NAC subunit (Egd2p) and two β -NAC subunits (Egd1p and Btt1p; Fig 1A). Two-hybrid assays indicated that the α - and β -subunits of NAC specifically recognize each other and that this interaction

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Fig 1 | Nascent chain-associated complex is a highly conserved protein complex. (A) Schematic representation of conserved domains in *Saccharomyces cerevisiae* (Sc) nascent chain-associated complex (NAC) subunits and archaeal NAC from *Methanosarcina acetivorans* (Ma). (B) Two-hybrid interaction of NAC subunits from different species. LexA and Gal4 fusions of the proteins indicated were tested in a yeast strain that lacked endogenous NAC genes. Hs, *Homo sapiens* (C) Phenotypes of NAC mutants and complementation by homologues from different species. Serial dilutions of yeast strains were spotted onto selective plates and growth was assayed at the indicated temperatures. WT, wild type. (D) Levels of Btt1p are elevated in the absence of Egdp1p. Western blots of yeast lysates were analysed with subunit-specific antibodies for Egdp1p and Btt1p. Actin, detected with α -actin antibodies, is shown as a loading control.

is conserved (Fig 1B). Yeast α -NAC interacts with either of the two yeast β -NAC subunits, but was not found to form homodimers in the two-hybrid system. Human α -NAC specifically interacts with either β -NAC subunit of yeast, but not with yeast α -NAC, indicating a high degree of conservation among orthologues from different species. In contrast to eukaryotes, archaeal genomes contain only a single NAC gene, homologous to both α - and β -NAC (Fig 1A). The recent crystal structure of NAC from *Methanothermobacter marburgensis* shows that archaeal NAC is homodimeric and combines features of both eukaryotic NAC subunits. This is reflected in our observation that archaeal NAC of *Methanosarcina acetivorans* (MaNAC) interacts with all subunits of yeast NAC (Fig 1B).

A phenotypical analysis of yeast mutants lacking the entire NAC or single subunits showed a temperature-sensitive (ts) growth defect at 37 °C (Fig 1C). We find that yeast mutants lacking either the α -subunit alone or both alternative β -subunits show a growth defect similar to cells lacking all subunits (Fig 1C), which is consistent with a previous report that a balanced ratio of α - to β -subunits is important for normal cell function (Reimann *et al*, 1999). The two β -subunits encoded by the *EGD1* and *BTT1* genes are functionally redundant, as only the double deletion $\Delta egd1\Delta btt1$ results in a ts phenotype (Fig 1C). Consistent with this finding, analysis of protein levels with subunit-specific antibodies showed a tenfold upregulation of the low-abundant Btt1p following the deletion of *EGD1* (Fig 1D). In addition to the interactions between the α - and β -subunits of different species, a complementation analysis showed that the NAC subunits are also functionally conserved. The ts phenotype of a yeast α -NAC deletion mutant could be complemented by human α -NAC, and archaeal NAC suppressed the phenotype at least partially, whereas bacterial TF did not complement the phenotype (Fig 1C). Thus, our results indicate a strong functional conservation among the eukaryotic α - and β -NAC subunits and a partial functional overlap with the archaeal NAC.

NAC and SRP bind to ribosomes at the subunit L25

Consistent with its known ribosome association (Reimann *et al*, 1999), NAC co-migrates with yeast polysomes in sucrose density gradients similar to other ribosome-associated, cytosolic factors, including the ribosome-associated complex (RAC; Gautschi *et al*, 2001) and the SSB-type Hsp70 proteins (Nelson *et al*, 1992). This co-migration is abolished by treatment with RNase A, which causes dissociation of polysomes (Fig 2A). Furthermore, ribosomes that are epitope tagged on subunit L4 co-precipitated substantial amounts of NAC and RAC as well as the 54 kDa subunit of SRP, which is a known interaction partner of ribosomes (Pool *et al*, 2002; Fig 2B,C). Remarkably, in comparison with ribosomes containing L4-9Myc as the only tagged subunit, another C-terminal 3HA (haemagglutinin) tag on L25 substantially reduced NAC binding to ribosomes, as shown by immunoprecipitation using L4-9Myc (Fig 2C).

According to the crystal structure of the large ribosomal subunit of *Haloarcula marismortui* (Ban *et al*, 2000), L25 is located in close proximity to the ribosomal exit tunnel for nascent polypeptides. The reduced binding efficiency of NAC to ribosomes tagged on L25 could be the result of steric hindrance. We therefore analysed L25 and three other subunits (L17, L26 and L35) located close to the exit tunnel as possible candidates for NAC-binding

Fig 2 | Nascent chain-associated complex and signal-recognition particle interact with the ribosomal protein L25. (A) Hsp70 (Ssb1/2p), ribosome-associated complex (RAC; Ssz1p) and nascent chain-associated complex (NAC; Egd2p) co-migrate with cycloheximide (CHX)-stabilized polysomes (L3) in sucrose density gradients (left). The interaction is lost after RNase treatment (right). Gradient fractions were analysed by western blotting with antibodies against the proteins indicated. (B) Co-immunoprecipitation of signal-recognition particle (SRP) and NAC subunits with ribosomes. Immunoprecipitation of ribosomes with a 9Myc tag at subunit L4 was carried out with c-Myc-specific antibodies and protein A-Sepharose as a control. Western blots of total cell extracts (TE; 10 μ g) and precipitates (IP; immunoprecipitates from lysates containing 200 μ g total protein) were analysed with the antibodies indicated. (C) An additional carboxy-terminal 3HA (haemagglutinin) epitope tag on L25 interferes with the binding of NAC to L4-9Myc-tagged ribosomes. Immunoprecipitations were carried out with c-Myc antibodies as in (B). Percentage values are from quantification of the amounts of co-precipitated NAC in the respective lanes and show that an additional epitope tag on L25 reduces the efficiency of co-precipitation to 30% of that without an additional epitope tag.

sites on the ribosome. In a yeast two-hybrid assay, only L25 was found to interact with β -NAC; no interaction was observed with α -NAC (not shown). Indeed, NAC contained in cell lysates bound specifically to an immobilized maltose-binding protein (MBP)-L25 fusion protein but not to MBP, as detected with antibodies against Egd1p, Egd2p and Btt1p. Similarly, SRP containing an HA-tagged Srp14p subunit bound to MBP-L25 (Fig 3A). In contrast, no direct interaction was seen between L25 and other ribosome-associated proteins, such as the RAC components Ssz1p and Zuo1p (Fig 3A). Together, these results identify L25 as a

Fig 3 | Isolated L25 specifically interacts with nascent chain-associated complex and signal-recognition particle. (A) Immobilized maltose-binding protein (MBP)-L25 was incubated with yeast lysate (200 µg protein). Western blots of total cell extracts (TE; 10 µg) and proteins bound to immobilized MBP-L25 (or MBP alone as control) (E) were analysed with antibodies against the proteins indicated. Eluates shown correspond to 200 µg of total extract. (B) The amino terminus of β-NAC is required for binding to L25. Interaction of purified NAC subunits with immobilized L25 (upper panel) and complex assembly of purified subunits were analysed by co-immunoprecipitation with anti-Egd2p antibodies (lower panel). Upper panel: I, input material (1 µg); MBP-L25, proteins eluted from immobilized MBP-L25; MBP, proteins eluted from MBP. Lower panel: I, input material (0.5 µg); IP, immunoprecipitations of assembled complexes with anti-Egd1p antibodies were analysed on Coomassie gels.

specific binding partner for NAC on ribosomes and confirm recent findings that L25 is a binding site for SRP (Pool *et al*, 2002).

L25-binding requires the amino-terminus of β-NAC

To study the interaction between NAC and L25 in more detail, we purified full-length and truncated forms of yeast NAC subunits and used individual subunits or reconstituted complexes for *in vitro* binding studies with immobilized MBP-L25. β-NAC (Egd1p and Btt1p) was found to bind to L25, whereas α-NAC (Egd2p) did not show a direct interaction (Fig 3B, upper panel; not shown), which was consistent with our two-hybrid data and with previous findings that only β-NAC interacts with ribosomes (Reimann *et al*, 1999; Beatrix *et al*, 2000). Trypsin treatment of β-NAC (Egd1p)

resulted in the production of a stable fragment that lacked amino-acid residues 1–31 (not shown). Recombinant Egd1Δ1–31p (Fig 1A) did not detectably interact with L25 (Fig 3B, upper panel), indicating that the N-terminal segment is involved in NAC binding to ribosomes. In contrast, Egd1Δ1–31p still interacted efficiently with the α-subunit Egd2p (Fig 3B, lower panel). Remarkably, neither overexpression of *EGD1* nor competition with purified protein reduced the binding of SRP to L25 (supplementary Fig 1A,B online; not shown), which suggests the existence of distinct, non-overlapping binding sites for the two proteins on L25. This is reminiscent of the situation in bacteria in which simultaneous binding of TF and SRP to L23 has been observed (Buskiewicz *et al*, 2004).

Purified NAC subunits have chaperone-like activity

Little is known about the functional properties of NAC. We therefore tested whether NAC is able to prevent protein aggregation, a functional property commonly associated with molecular chaperones. The aggregation of the model protein rhodanese on dilution from denaturant was followed by measuring light scattering. Both reconstituted yeast NAC and the individual subunits strongly reduced the aggregation of rhodanese (Fig 4A,B). This effect was concentration dependent, with little or no measurable aggregation occurring at a five- to tenfold molar excess of NAC relative to the denatured protein, whereas the control protein bovine serum albumin (BSA) showed no effect. A concentration of 5 µM NAC was as efficient in aggregation prevention as 1 µM of the chaperonin GroEL, which was used as a positive control (Fig 4B). Dimerization of NAC subunits was not required for this chaperone-like activity, as the individual subunits Egd1p and Egd2p alone also prevented rhodanese aggregation with similar or only slightly reduced efficiency (Fig 4A).

SRP and α-NAC interact directly

In addition to the identification of L25 as a shared binding partner for NAC and SRP, our experiments showed a direct interaction between α-NAC and SRP. In a two-hybrid analysis, yeast Srp54p specifically interacted with yeast and human α-NAC, but not with β-NAC. Moreover, a positive two-hybrid signal was observed between archaeal NAC from *M. acetivorans* and either archaeal SRP or yeast Srp54p (Fig 5A), which indicated that the interaction between NAC and SRP had been conserved in evolution. After treatment of cell extracts with the crosslinker dithiobis (sulfo-succinimidyl propionate; DTSSP), SRP with an epitope tag on the 14 kDa subunit (or on Srp54p) co-precipitated Egd2p but, surprisingly, not β-NAC (Egd1p; Fig 5B; not shown). Co-precipitation of SRP and α-NAC was also detectable in the absence of crosslinker, although with lower efficiency (not shown), which suggests that the interaction is rather transient. Either SRP only interacts with free Egd2p, not in complex with Egd1p, or binding of Egd2p to Srp54p weakens the interaction between Egd2p and Egd1p. Moreover, the interaction between α- and β-NAC may be dynamic and the separate subunits may fulfil other functions. Previous reports on the function of individual NAC subunits in transcriptional regulation and apoptosis support this possibility (Parthun *et al*, 1992; Bloss *et al*, 2003).

The direct interaction between NAC and SRP is consistent with the proposal that NAC can function as a regulator of protein translocation into the ER, by preventing the interaction of SRP with

Fig 4 | Nascent chain-associated complex has chaperone-like activity. (A) Nascent chain-associated complex (NAC) can suppress protein aggregation. Denatured rhodanese (0.5 μ M final concentration) was diluted into buffer without or with a tenfold molar excess of NAC, NAC subunits or BSA (upper panel) and aggregation was monitored by light scattering. *Escherichia coli* GroEL (1 μ M) was used as a positive control. (B) The concentration dependence of the prevention of rhodanese aggregation is shown for a four-, six-, eight- and tenfold molar excess of NAC with 1 μ M GroEL as a control.

non-secretory proteins and thus their mistargeting (Lauring *et al*, 1995). Cells accumulating non-native proteins in the ER specifically activate a stress-response pathway known as the unfolded protein response (UPR; Ma & Hendershot, 2001). The expression of a sensitive UPR-*lacZ*-reporter construct (Cox *et al*, 1997) was used to test whether loss of NAC function induces the UPR (Fig 5C). The complete deletion of NAC resulted in a moderate induction of the UPR reporter at 30 $^{\circ}$ C. However, at 37 $^{\circ}$ C, that is, under conditions in which the NAC deletion strain exhibits a pronounced growth defect (Fig 1C), a strong increase in the UPR signal was observed compared with wild-type cells (Fig 5C). Depletion of NAC from translating ribosomes has been shown to result in SRP-mediated mistargeting of non-secretory polypeptides *in vitro* (Lauring *et al*, 1995; George *et al*, 1998; Beatrix *et al*, 2000), and this effect would plausibly explain the induction of the UPR observed following NAC deletion *in vivo*. The direct interaction between α -NAC and SRP described here may have a role in avoiding such mistargeting.

DISCUSSION

We have shown that the ribosomal protein L25 mediates the association of both NAC and SRP with the translation machinery. Thus, both factors dock onto the ribosome in close proximity to each other and to the exit site for newly synthesized polypeptides. This location would not only facilitate the interaction of NAC and SRP with nascent chains but also allow for functional crosstalk

Fig 5 | Nascent chain-associated complex interacts with signal-recognition particle and the loss of its function results in induction of the unfolded protein response. (A) Two-hybrid interaction of Srp54p and nascent chain-associated complex (NAC) subunits from different species (yeast, human and archaea). LexA and Gal4 fusions of the indicated proteins were analysed in a yeast strain lacking endogenous NAC genes. (B) Co-immunoprecipitation of signal-recognition particle (SRP) and α -NAC. SRP was immunoprecipitated from DTSSP-treated lysates of a yeast strain carrying a 3HA (haemagglutinin) tag on the 14 kDa subunit of SRP and precipitates were analysed for co-precipitation of Egdp and Egdp by Western blotting. TE, total cell extract; IP, immunoprecipitation. (C) Unfolded protein response (UPR) assay of wild-type (WT) cells and NAC mutants. β -Galactosidase (β -Gal) activity of the UPR-reporter construct pJ31 carrying the UPR element of the *KAR2* promoter inserted into a disabled *CYC1* promoter upstream of the *lacZ* gene (Cox *et al*, 1997) was assayed on plates and in liquid assays. Data are from six independent experiments; β -Gal units of wild-type cells at 30 $^{\circ}$ C were set to 1. As a positive control for UPR induction, cells were treated with tunicamycin (Tm; 1 μ g/ml) at 30 $^{\circ}$ C.

between the two components. Indeed, α -NAC and the 54 kDa subunit of SRP are found to interact directly. Furthermore, NAC deletion in yeast leads to a pronounced growth defect at 37 °C and an induction of the UPR pathway, which is indicative of the accumulation of misfolded proteins in the ER. Among other possibilities, these findings are consistent with the proposed role for NAC in ensuring that SRP binds exclusively to secretory proteins, thereby preventing the mistargeting of non-secretory proteins to the ER. Although the exact role of NAC in protein biogenesis and quality control remains to be explored, the ability of purified NAC to prevent the aggregation of a denatured model protein suggests that NAC has chaperone-like properties.

In the light of recent findings that, in bacteria, both TF and SRP interact with the ribosomal protein L23 (Kramer *et al*, 2002; Gu *et al*, 2003), the homologue of eukaryotic L25, our results indicate that the basic mechanism by which the translation machinery is physically linked to downstream processes of protein biogenesis is conserved between prokaryotes and eukaryotes. However, despite this commonality, there are interesting differences between the bacterial and eukaryotic systems. While L23/L25 has been maintained as the docking site, TF is restricted to bacteria, whereas NAC is found in archaea and eukarya. NAC and TF have no apparent structural similarity (Ferbitz *et al*, 2004; Ludlam *et al*, 2004; Spreter *et al*, 2005) and it seems unlikely that NAC fulfils a general, TF-like chaperone function in protein folding. For example, whereas the combined deletion of TF and DnaK (Hsp70) in *Escherichia coli* causes synthetic lethality at growth temperatures above 30 °C (Deuerling *et al*, 1999; Teter *et al*, 1999), the combined deletion of NAC and other principal cytosolic chaperones in yeast does not result in a synthetic phenotype (Gautschi *et al*, 2002; Siegers *et al*, 2003). Moreover, TF cannot compensate for the loss of NAC function (Fig 1C). Thus, rather than being a general chaperone for *de novo* folding, NAC may have a regulatory role in protein quality control that involves chaperone-like properties. The interaction of NAC and SRP, conserved from archaea to mammalia, may be related to the proposed role of NAC in preventing inappropriate protein targeting by blocking ribosome docking to Sec61 (Lauring *et al*, 1995). A direct interaction with SRP was not found for TF, which regulates protein export by competing with SRP for binding to secretory proteins (Lee & Bernstein, 2002; Eisner *et al*, 2003). Further studies will have to show the functional consequence of the interaction between NAC and SRP, and other possible roles for NAC in quality control that may be related to the presence of the UBA domain in α -NAC.

METHODS

Strains and genetic manipulations. Yeast methods and media were as described (Siegers *et al*, 2003). Yeast strains were derivatives of YPH499 (Sikorski & Hieter, 1989). Yeast, archaeal and human genes were amplified from chromosomal DNA, or a human brain complementary DNA library and cloned into yeast expression and two-hybrid vectors.

Two-hybrid and β -galactosidase assays. Two-hybrid studies were conducted with the reporter plasmid pSH18-34 (Invitrogen GmbH, Karlsruhe, Germany) in a yeast strain that lacked endogenous NAC genes. β -Galactosidase assays of permeabilized cells and calculation of specific activities were carried out as described previously (Knop & Schiebel, 1997).

Protein purification. Recombinant MalE-L25 was obtained using the pMAL Protein Fusion and Purification System (New England Biolabs GmbH, Frankfurt am Main, Germany), and NAC subunits were produced with removable N-terminal hexahistidine tags using the pProEX Expression System.

Immunoprecipitations, *in vitro* binding assays and western blotting. Specific antibodies or pre-immune sera were crosslinked to protein A-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden), according to standard procedures. Lysis, immunoprecipitation and western blotting were carried out as described (Siegers *et al*, 2003). Briefly, cytosolic extracts were prepared by agitating the cells with glass beads. For crosslinking, lysates were treated with the homobifunctional crosslinker DTSSP (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Lysates were cleared by centrifugation (20,000g, 4 °C, 15 min). Supernatants were incubated in the presence of 1% Tween 20 with antibodies coupled to protein A-Sepharose beads or immobilized MalE fusion proteins for 2 h at 4 °C on a rotating platform. For *in vitro* binding experiments with purified proteins, equimolar amounts of the proteins were used. Beads were washed extensively with lysis buffer containing 1% Tween 20 before SDS-polyacrylamide gel electrophoresis. For western blots, 10 μ g of the total cell extract was analysed and 200 μ g protein was used for each precipitation experiment. Western blotting was carried out using the luminescence-based ECL system (Pharmacia).

Miscellaneous. Fractionation of ribosomes from yeast lysates was carried out as described (Siegers *et al*, 2003). Prevention of rhodanese aggregation was assayed by light scattering at 320 nm (Weber & Hayer-Hartl, 2000). Denatured bovine rhodanese (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was diluted 100-fold into buffer alone or into buffer containing various amounts of test proteins (Fig 4).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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