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Nascent-polypeptide-associated complex

S. Rospert[a,*], Y. Dubaquié[b] and M. Gautschi[a]

[a] Max Planck Research Unit for Enzymology of Protein Folding, Weinbergweg 22, 06120 Halle (Germany)
[b] Bristol-Myers Squibb, Pharmaceutical Research Institute, H13-03 P.O. Box 4000, Princeton, New Jersey 08543-4000 (USA), e-mail: rospert@enzyme-halle.mpg.de

Abstract. Nascent-polypeptide-associated complex (NAC) is a heterodimeric complex which can reversibly bind to eukaryotic ribosomes. NAC is located in direct proximity to newly synthesized polypeptide chains as they emerge from the ribosome. Although its function is thought to be conserved from yeast to humans our current knowledge about what NAC actually does in a living cell is incomplete. It has been suggested that NAC is a (i) dynamic component of the ribosomal exit tunnel, providing a shield for nascent polypeptides, (ii) negative regulator of translocation into the endoplasmic reticulum and (iii) positive regulator of translocation into the mitochondria. However, none of these hypotheses is generally accepted. Moreover, the individual subunits of NAC have been implicated in processes related to transcription rather than translation, and it is currently under debate whether NAC might be a protein of dual function. This review attempts to summarize the data from different fields and to discuss the partly controversial results in a common context.

Key words. ER translocation; translational repression; bicaudal; chaperone; nascent polypeptide.

Introduction

Ribosomes are molecular machines that synthesize polypeptides. They mainly consist of RNA, and even the active site of the peptidyl-transferase center is almost devoid of protein. Core ribosomal proteins seem predominantly involved in stabilizing the ribosomal RNA (rRNA) structure [1, 2]. The term 'molecular chaperone' describes various ubiquitous protein families that have the ability to interact with nonnative polypeptide substrates [3]. There is an increasing number of proteins that share at least some properties with classical chaperones and are specifically associated with ribosomes. NAC (nascent-polypeptide-associated complex) is a dimeric complex that was identified in higher eukaryotes as a ribosome-associated factor in close proximity to nascent chains [4] (compare table 1 for the nomenclature used throughout the text). Like a typical chaperone, NAC interacts with unfolded polypeptide chains independent of their amino acid sequence [4, 5]. What ribosome-bound NAC does exactly in vivo is only poorly understood and controversial. In addition, the individual subunits of NAC have been implicated in different processes related to transcriptional regulation. Below we will first summarize the findings related to potential transcription-related functions of the individual NAC subunits, and subsequently discuss our current knowledge of dimeric NAC acting as a ribosome-associated factor cotranslationally.

* Corresponding author.

Individual subunits of NAC

Early experiments suggest a role of βNAC in the initiation of transcription

βNAC, originally termed BTF3 (Basic Transcription Factor 3), was purified from HeLa cell extracts using run-off transcription as an in vitro assay [6, 7]. βNAC does not bind DNA by itself; however, complex formation of βNAC with RNA polymerase II has been reported [7, 8]. Based on these findings, it was claimed that βNAC is required for RNA polymerase II-dependent transcriptional initiation. Later, it was found that the gene encoding βNAC gives rise to two splicing variants – β_2NAC (BTF3a) and β_1NAC (BTF3b), an N-terminally truncated version, shortened by 44 amino acids [8, 9] (fig. 1A). β_1NAC can also bind RNA polymerase II; however, it

Table 1. Abbreviations for the two NAC subunits.

	Saccharomyces cerevisiae	Mammals
	αNAC	
αNAC	Egd2p (Enhancer of Gal4 DNA binding)	αNAC (NACA) skNAC (splicing variant of αNAC)
	βNAC	
β_1NAC	Egd1p (Enhancer of Gal4 DNA binding)	BTF3b (*B*asic *t*ranscription *f*actor 3b)
β_2NAC		BTF3a (*B*asic *t*ranscription *f*actor 3a)
β_3NAC	Btt1p (Btf Three)	–

does not stimulate transcriptional activation [8]. The original idea that β_2NAC is identical to the general transcription factor TFIIB was later corrected [10]. In the same year, a careful investigation defined the minimal factors required for transcription by mammalian RNA polymerase II. The study concluded that active preparations of general transcription factors and RNA polymerase II are lacking β_2 or β_1NAC [11]. Moreover, addition of pure βNAC was without effect on transcription in vitro, indicating that βNAC was dispensable for RNA polymerase II function. Earlier results were explained by trace amounts of general transcription factors in βNAC preparations [11, 12]. Considering the more recent work on NAC and the lack of in vivo evidence, we view a role for βNAC in transcriptional initiation as questionable. However, this view is debated, and a number of publications addressing different aspects of βNAC presume its activity as a transcription factor [13–18].

A potential role for the yeast homolog of β_1NAC in transcriptional regulation

The yeast homolog of β_1NAC was discovered using a gel retardation assay to monitor the activity stabilizing a complex between Gal4p and DNA [19]. The purified active fraction contained two polypeptides of 21 and 27 kDa molecular mass. The 21-kDa protein was cloned, and the gene was named *EGD1* (Enhancer of GAL4 DNA binding). Later, the copurifying 27-kDa band turned out to be the yeast αNAC homolog and was termed *EGD2* [20]. The *egd1* null caused no significant effects on growth, and only a minor transient defect in *GAL10* induction when cells were shifted from glucose to galactose [19]. In 1994 a second homolog of β_1NAC was identified in yeast and termed *BTT1* (BTf Three) [21] (fig. 1A). Hu and Ronne confirmed a transient twofold decrease of *GAL10* induction previously described for the Δ*egd1* strain [19]. No such effect was observed for Δ*btt1* [21]. The yeast strain disrupted in both *EGD1* and *BTT1* was still viable even on galactose, and did not display decreased *GAL10* induction. Instead, Δ*egd1*Δ*btt1* showed significant increase not only in the expression of several galactose-regulated genes but also of constitutively expressed genes such as actin (*ACT1*) [21]. Thus, loss of both yeast βNAC homologs enhances the expression of several genes, including the *GAL* genes, while loss of *EGD1* alone has a small negative effect on the *GAL* genes only. The reason for this puzzling observation is unknown. A possible explanation is provided by the following observation. Yeast β_1NAC (Egd1p) as well as yeast β_3NAC (Btt1p) form a complex with αNAC (Egd2p) in vivo. Cells lacking both β_1NAC and β_3NAC show a growth defect at 37 °C. This temperature sensitivity, however, is cured when in addition *EGD2* is disrupted in the Δ*egd1*Δ*btt1* background [22]. The result suggests that in the absence of its partner subunits, αNAC negatively affects growth of yeast. Possibly the induction of genes observed in the absence of both βNAC homologs does not reflect a regulatory role of βNAC but rather is due to a toxic effect of 'monomeric' αNAC.

The in vivo role of βNAC in higher eukaryotes

The gene encoding the mouse homolog of βNAC was disrupted in mouse embryonic stem cells. Mice homozygous for the mutant allele die soon after implantation, around day 6. Thus, βNAC is dispensable through preimplantation stages and implant, but is essential for early postimplantation development [13]. The mechanism by which βNAC influences mouse development is unknown. In vivo evidence for a role of β_1NAC in translational regulation comes from a study by Markesich and co-workers. In *Drosphila* some messenger RNAs (mRNAs) are translationally repressed when not localized to the posterior during development. One of them is the *nos* transcript, which encodes Nanos, a protein that directs posterior cell fates (reviewed in [23]). It was recently shown that *nos* mRNA is associated with polysomes, and translational repression is achieved by a mechanism downstream of translation initiation [24]. *Drosophila* β_1NAC, encoded by *bicaudal,* is a widely expressed gene. *Bicaudal* was the first *Drosophila* mutation with clear effect on embryonic pattern formation [25]. At its most extreme, the mutation produces embryos in which head and thorax are missing and are replaced with mirror-image duplications of abdominal segments. Like the mouse homolog,

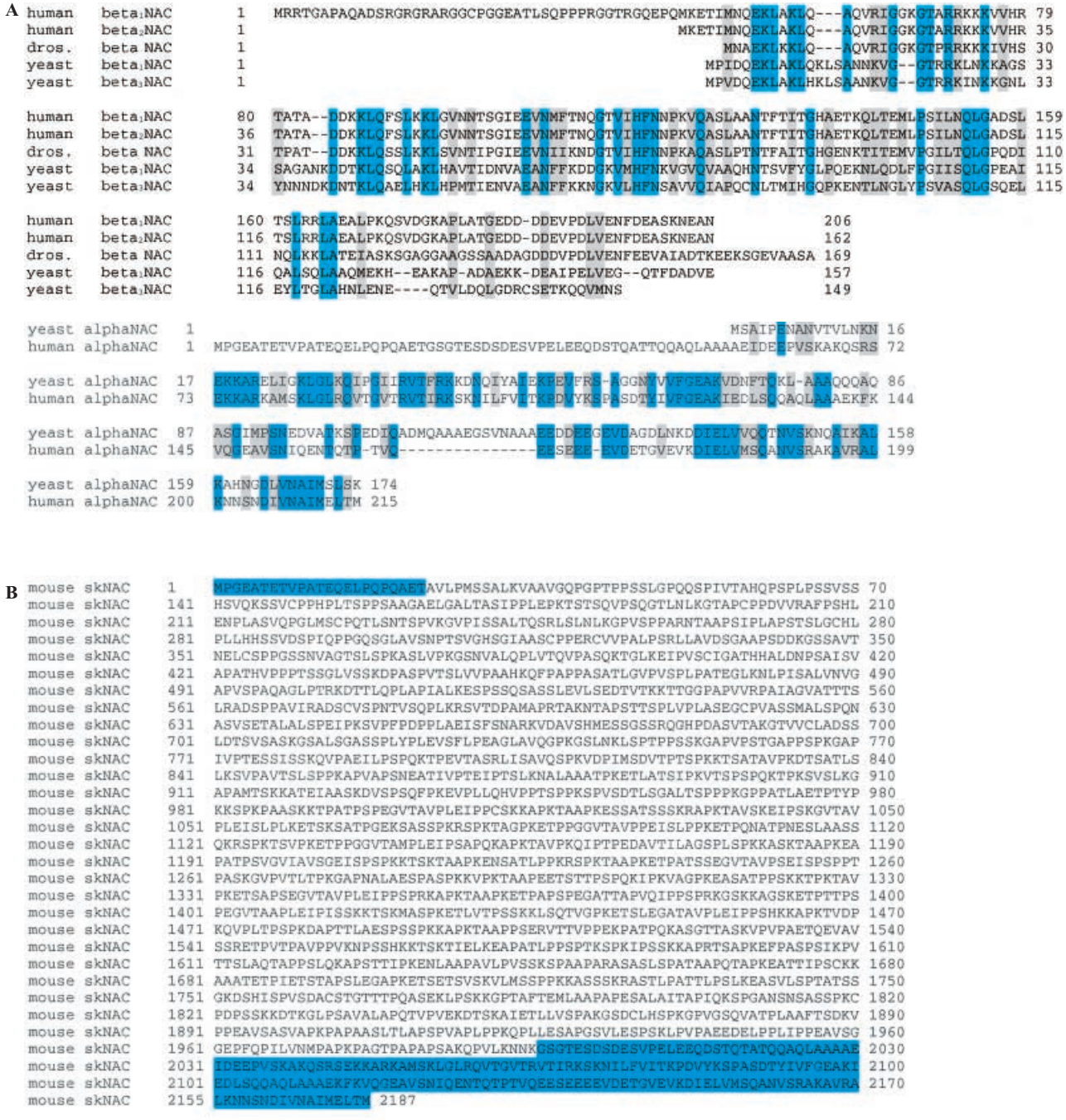

Figure 1. (*A*) βNAC is a protein conserved from yeast to humans. In mammalian cells BTF3 homologs form a gene family [9]. Some of the mammalian homologs in the database contain N-terminal extensions. One of the N-terminally extended versions, β_2NAC, is a splicing variant of β_1NAC. *Drosophila* expresses only the shorter version corresponding to mammalian β_1NAC [12]. Yeast contains two genes encoding proteins homologous to β_1NAC – *EGD1* and *BTT1* [19] [21]. (*B*) αNAC is conserved from yeast to humans [20]. skNAC arises from differential splicing. The alternatively spliced exon is of unusually large size: skNAC has a molecular mass of 221 kDa, compared with 23 kDa of the αNAC protein [26]. The mouse αNAC sequence is labeled in blue. Homologs of βNAC or αNAC in eubacteria have so far not been reported. Distant homologs of αNAC are present in archaebacteria.

bicaudal produces 100% embryonic lethality; most embryos die in late embryogenesis [12]. Markesich and co-workers show that *bicaudal* results in ectopic translation of *nos* mRNA, and suggest that *bicaudal*'*s* effect might be a direct consequence of loss of β_1NAC function at the ribosome, resulting in a release of translational repression.

A role for αNAC as transcriptional coactivator?

First evidence for a role of αNAC-homologs in transcription was obtained with a muscle-specific isoform, termed skNAC, which was identified in mouse [26, 27] (fig. 1B). Only skNAC displays transcriptional activating capacity; the murine myoglobin promoter was identified as a natural promoter responsive to skNAC. αNAC was not active in stimulating transcription from reporter constructs; neither did αNAC competitively inhibit transcriptional activation by skNAC [26]. Munz and co-workers, who showed that skNAC is involved in the repair process of injured muscle cells, confirmed a specific role for the large splicing variant skNAC [28].

In two back-to-back publications the group of St-Arnaud presented evidence that αNAC, although not a transcription factor on its own, might function as a developmentally regulated, bone-specific transcriptional coactivator [29, 30]. The main results in support of this conclusion are (i) high expression of αNAC in differentiated osteoblasts at the ossification centers [30]; (ii) partial translocation of αNAC to the nucleus of mouse osteoblasts when cells are arrested in G_o/G_1; (iii) increased GAL4/VP-16-mediated mRNA synthesis in cells transiently transfected with αNAC [29]; (iv) interaction of αNAC with the transcriptional activator c-Jun and the TATA box-binding protein TBP in vitro [29, 30] and (v) binding of αNAC to a specific oligonucleotide in vitro [29]. In view of these results, a recent review favors a role of αNAC as a transcriptional coactivator and raises the question whether the same protein could truly exert dual function [17]. On the other hand, recent work questions αNAC´s role as a transcriptional coactivator. High expression of αNAC in mammals is not restricted to osteoblasts. The NAC complex was shown to be present in the range of 3–10 µM in a variety of tissues [31], and the relocation of mammalian αNAC from the cytosol to the nucleus upon starvation could not be reproduced [32]. In yeast nuclear import of the αNAC – and also βNAC – can only be observed if one of the subunits is overexpressed. The NAC subunits are thought to enter the nucleus in order to assemble with the ribosomal subunits [33]. Binding of αNAC to nucleic acids was confirmed; however, advanced studies suggest that binding is rather independent of the DNA sequence and that αNAC can also bind to rRNA or transfer RNA (tRNA) [32]. αNAC's high affinity for a variety of nucleic acid resembles zuotin, another ribosome-associated protein. Zuotin was initially found to be a protein with high affinity for Z-DNA [34], but the protein was also shown to bind to tRNA [35] and rRNA [36].

Differing results regarding the in vivo role of αNAC most likely reflect different experimental approaches. St-Arnaud and co-workers – who favor αNAC involved in transcriptional regulation – have mainly studied αNAC's properties in the absence of βNAC in vitro, or after overexpression of αNAC in cell culture [29, 30]. Investigators favoring a ribosomal function of αNAC have studied the protein as one subunit of a dimeric complex, that is in the presence of equal amounts of βNAC [4, 12, 22, 31–33, 37–44]. However, there might be individual functions for αNAC and the α/βNAC complex in vivo. This view is supported by the finding that mouse αNAC can be active as a transcriptional coactivator in vivo. However, it loses its activity when β_1NAC (BTF3b) is coexpressed [29]. In yeast the presence of 'monomeric' αNAC is toxic for cell growth at 37 °C [22] (see also above). These findings might reflect a regulatory role of 'monomeric' αNAC in transcriptional regulation. Whether αNAC is involved in transcription in vivo, and whether a transcriptional role of αNAC may be regulated by dimerization with βNAC, remains to be determined.

The dimeric NAC complex: an adaptor between ribosomes and the cellular folding and transport machineries?

Localization and oligomeric structure of NAC

The dimeric complex of NAC was originally purified from bovine brain [4]. The vast majority of α- and βNAC subunits are involved in complex formation [32]. Monomeric subunits may exist, but their concentration would be below the detection limit. Similar results were obtained for the yeast homologs [22]. In mammals both splicing variants of the β subunit – β_1 and β_2 – form a stable complex with αNAC. The α/β_2 complex has not been characterized in vitro, but it is thought to have properties similar to the α/β_1NAC complex [32].

In yeast the bulk of NAC can be isolated in a complex with cytosolic ribosomes [22, 42, 45]. Stable binding of NAC to the ribosome requires the presence of a β subunit [22, 32]. The concentration of the yeast αNAC/β_1NAC complex was estimated to be roughly equimolar to ribosomes, while the αNAC/β_3NAC complex is 100-fold less abundant [22, 42]. In mammals the concentration of NAC in different tissues is in the range of 3–10 µM [31]. Assuming the number of ribosomes per cell to be roughly 6×10^6 [46] and an average cell diameter of 30 µm, the concentration of ribosomes in a eukaryotic cell is ~1 µM. In agreement with this estimate, the concentration of ribosomes and NAC in reticulocyte lysate is 75–100 nM

and 1–2 μM, respectively [44]. Thus, in a yeast cell each ribosome seems to carry one NAC molecule, while in higher eukaryotes more than one NAC molecule could potentially bind [31, 40]. This high concentration of NAC, in combination with the finding that NAC protects very short nascent chains from proteolysis and can be cross-linked to nascent chains of only 17 amino acids, led to the hypothesis that NAC is an integral component of the ribosomal exit tunnel [40, 47]. In view of the topology of the archebacterial ribosomal exit tunnel, a potential structural participation of NAC remains unclear [48].

Interaction between NAC and ribosome-bound nascent chains

Several studies based on cross-link approaches have shown that both subunits of mammalian and yeast NAC are located in close proximity to ribosome-bound nascent chains [4, 22, 32, 44, 49]. These cross-link experiments suggest that NAC does not display pronounced sequence specificity but ubiquitously binds to nascent chains in a distance of 17–100 amino acids from the ribosomal peptidyl transferase center [4, 40]. NAC's affinity for unfolded proteins in the absence of the ribosome is low. Binding to proteins after release from the ribosome has not been demonstrated so far [4, 49].

NAC: a controller of proper translocation to the ER?

Different mechanisms are involved in maintaining specificity of protein translocation into the ER (endoplasmic reticulum). Most important, specificity is guaranteed by a targeting signal termed the signal sequence, contained in the N-terminus of proteins destined to the ER. SRP (signal recognition particle), a cytosolic ribonucleoprotein complex, recognizes signal sequences and is essential for efficient cotranslational translocation of proteins to the ER (for reviews on SRP and its function see [50–53]). In a subsequent step the signal sequence is recognized by the Sec61 complex, the protein-conducting channel in the lipid bilayer. This proofreading leads to a tight junction between signal sequence-bearing ribosome-nascent chain complexes (RNCs), and Sec61 and is thought to prevent signalless RNCs from being translocated [54].

One aspect of the ER-translocation machinery is difficult to reconcile with proper sorting. The Sec61 complex functions not only as the translocation channel but is also a high-affinity ribosome receptor [55, 56]. Ribosome binding to the Sec61 complex occurs via the rRNA of the large ribosomal subunit [57–59]. This raises the question of how vacant ribosomes or ribosomes translating cytosolic proteins are prevented from displacing signal sequence-bearing RNCs from the Sec61 complex. Furthermore, signal sequence-independent binding of RNCs to the Sec61 complex might result in aberrant transport of cytoplasmic proteins into the lumen of the ER, a phenomenon that can be observed in vitro [4, 39, 54].

When NAC was identified as a ribosome-bound factor, it was proposed that NAC, besides SRP, was the second cytosolic factor essential for faithful targeting of proteins to the ER. This hypothesis was supported by in vitro experiments indicating that in the absence of NAC (i) ribosome-bound nascent chains can be cross-linked to SRP independent of whether they bear a signal sequence, (ii) RNCs bind to ER membranes independent of the presence of a signal sequence or SRP and (iii) signalless RNCs can be translocated across the ER membrane, albeit inefficiently [4, 31, 32, 39, 41, 60]. Based on these data it was proposed that NAC serves a dual function in ensuring proper translocation of proteins to the ER. First, binding of NAC to signalless nascent chains would be required to prevent them from interaction with SRP. Second, binding of NAC to the so-called ribosomal M site (membrane attachment site) of RNCs or ribosomes would prevent their interaction with the protein conducting channel in the ER membrane. However, the view that NAC is a 'global ribosome-binding prevention factor' and SRP 'a regulatory protein rather than a targeting factor' [60] is not generally accepted.

The hypothesis that SRP requires NAC to accomplish specific binding to signal sequences was originally challenged by Powers and Walter. This study dissected the interaction of SRP with RNCs into a salt-sensitive interaction between SRP and the ribosome and a salt-resistant interaction between SRP and a signal sequence [61]. NAC was shown to prevent the salt-sensitive binding of SRP to signalless RNCs. As NAC also prevents SRP binding to vacant ribosomes, the two particles are unlikely to compete for binding to nascent chains. More likely, SRP and NAC compete for the same site, possibly the M site, on the ribosome [41, 61]. This competition might facilitate SRP sampling of nascent chains for signal sequences, which is achieved by binding and release of SRP from the ribosome during the elongation process, and NAC might increase the rate at which SRP dissociates from signalless RNCs. However, it is presently unknown how exactly NAC would influence SRP function [61, 62]. Whether NAC influences ribosome binding to the ER membrane is also a matter of debate. Two groups independently reported that NAC does not interfere with binding of ribosomes or signalless RNCs to the Sec61 complex [43, 44]. Both groups also presented evidence that SRP-dependent binding of RNCs to the protein-conducting channel was insensitive to the presence of an excess of vacant ribosomes or signalless RNCs, indicating that SRP is a positive effector sufficient to account for selective targeting of signal sequence-bearing RNCs [43, 44].

The conflicting data are most likely due to differences in the experimental setup of the in vitro experiments. Incomplete detachment of endogenous membrane-bound ribosomes from microsome preparations is discussed as a reason for misleading results [43, 44]; however, the Wiedmann group [31] later addressed this objection. On the other hand, the concentration of NAC used in the experiments varied, and high concentrations of NAC might be critical for the outcome of the experiment [31]. Another possibly important difference might be the use of purified NAC versus cytosol containing NAC in combination with high-salt-treated RNCs for binding or translocation experiments. Maybe the cytosol contains other ribosome-binding proteins besides NAC and SRP that influence the properties of the RNCs. However, data obtained with low-salt RNCs generated in yeast translation extracts derived from NAC-deletion strains argue against this possibility [38]. The majority of these observations have been generated by locking nascent chains on the ribosome, stripping off loosely bound proteins and subsequently adding back purified factors. Although very powerful, this artificial in vitro system might have finally reached its limits. Most likely, in vivo studies or novel, more physiological in vitro approaches will solve the current contradiction.

Whether NAC affects translocation into the ER in vivo has so far only been addressed in yeast [22, 38, 42]. As outlined above, yeast possess one αNAC (Egd2p) homolog and two homologs of βNAC (Egd1p and Btt1p). Cells lacking all three subunits are viable and do not display any significant phenotype [22]. In agreement with the lack of a phenotype, signalless invertase is not mistranslocated into the ER in the absence of NAC [22, 38]. The results suggest that in yeast either NAC is not involved in sorting of proteins to the ER, or a redundant system can complement NAC's absence.

NAC: a factor stimulating translocation into mitochondria?

Recent experiments raised the question whether NAC might be involved in targeting proteins to the mitochondria of yeast. Initial experiments were performed with a yeast strain lacking both αNAC and a protein termed Mft52 (Mft1p). This strain shows an increased tendency to lose mitochondrial DNA; however, it does not become respiratory deficient [42]. It also displays a defect in the delivery of a synthetic fusion protein targeted to the mitochondria, but translocation of 'natural' mitochondrial precursor proteins remained largely unaffected [42]. Later it was shown that Mft52 is a subunit of a complex that connects transcriptional elongation to mitotic recombination and, most likely, is not involved in targeting to the mitochondria [63]. Therefore, mitochondrial defects observed in the strain lacking αNAC and Mft52 may have been indirect.

Yeast strains lacking functional NAC do not display a mitochondrial phenotype. Defects of mitochondrial protein translocation have not been observed in vivo ([22] and our own unpublished data). However, an in vitro translocation assay revealed a positive effect of NAC on the translocation of a mitochondrial precursor protein trapped in a ribosome nascent-chain complex [37]. We think that this stimulatory effect of NAC on a mitochondrial precursor protein in vitro does not correspond to a mitochondrial targeting function of NAC in vivo for the following reasons. First, there is no evidence for a 'NAC receptor' – resembling the SRP receptor of the ER membrane – in the mitochondrial membrane [37]. Second, the effect of NAC on the ribosome-bound mitochondrial precursor is not unique – yeast RAC (ribosome-associated complex), consisting of the Hsp70 homolog Ssz1p and the Hsp40 homolog zuotin, also stimulates translocation into mitochondria [45, 64]. The finding that NAC affects a nascent chain in a manner similar to RAC, a classical chaperone, is so far one of the few indications that NAC might really possess chaperone-like properties (suggested earlier by [4, 65, 66]). NAC, like RAC, might be able to present a ribosome-bound mitochondrial precursor in a conformation that can be efficiently recognized by the mitochondrial import machinery. In vivo, this ability does not seem to be essential for mitochondrial protein translocation. Even a yeast strain lacking both NAC and RAC does not display mitochondrial defects [45]. Most likely, translocation into mitochondria proceeds mainly via the posttranslational pathway and does not critically depend on ribosome-bound chaperones [67].

What does NAC do in vivo?

Up to now, the in vivo function of NAC has remained obscure. Clearly, NAC can bind nascent polypeptide chains. By that mechanism it might shield them against improper folding or unwanted interaction with other proteins. It is an attractive hypothesis that NAC is involved in the delivery of newly synthesized proteins to the cytosolic protein-folding machinery or to the different translocation systems in the eukaryotic cell [66]. However, up to now convincing evidence for such a role is lacking. NAC has been compared to trigger factor, a prokaryotic ribosome-bound prolyl-isomerase [5]. Again, there is no evidence that NAC would functionally cooperate with a chaperone of the Hsp70 class like trigger factor does [68, 69]. The in vivo results obtained in *Drosophila* might yet suggest another scenario: NAC might not only bind nascent chains but, through its interaction with the ribosome, may also actually regulate further translation of growing polypeptides. By that mechanism it could be an important regulator of developmental processes, as suggested by the severe phenotypes observed in mouse and fly.

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