The α and β Subunit of the Nascent Polypeptide-associated Complex Have Distinct Functions*

Birgitta Beatrix‡, Hideaki Sakai§, and Martin Wiedmann‡¶

From the ‡Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 and the §Department of Pharmacology, School of Dentistry, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852, Japan

Nascent polypeptide-associated complex (NAC) is probably the first cytosolic protein to contact nascent polypeptide chains emerging from ribosomes. In this way NAC prevents inappropriate interactions with other factors. Eventually other factors involved in targeting and folding, like the Signal Recognition Particle or cytosolic chaperones, must gain access to the nascent chain. All NAC preparations to date consist of two copurifying polypeptides. Here we rigorously show that these two polypeptides, termed α- and βNAC, form a very stable complex in vivo and in vitro and that a functional complex can be reconstituted from the individual subunits. A dissection of the contributions of the individual subunits to NACs function revealed that both subunits are in direct contact with nascent polypeptide chains on the ribosome and that both contribute to the prevention of inappropriate interactions. However, βNAC alone directly binds to the ribosome and is sufficient to prevent ribosome binding to the endoplasmic reticulum membrane.

Nascent polypeptide-associated complex (NAC) is a very abundant cytosolic protein, which is involved in cotranslational targeting of polypeptides to the endoplasmic reticulum (ER) membrane. The intracellular NAC concentration varies only slightly in different tissues, ranging from 3 to 10 μM (1). NAC is highly conserved among eukaryotes, but up to now no functional prokaryotic homolog has been described. The importance of NACs in vivo function is emphasized by early embryonically lethal phenotypes of NAC mutants in mice and fruit flies (2, 3). Recently it was also shown that yeast NAC, although not essential for growth (4), is involved in the import of proteins into mitochondria (5, 6).

NAC was originally identified as a ribosome-associated protein when we set out to probe the molecular environment of growing polypeptides on the ribosome using a photo-cross-linking approach (7). With this technique we were able to show that NAC can interact with regions of nascent chains as close as 17 amino acids to the peptidyl transferase center. Additionally, it was shown by protease protection that NAC functions as a dissociable wall of a ribosomal tunnel through which the growing polypeptide emerges (8). Cycles of binding and releasing NAC expose the polypeptide to the cytosol in “quantal units” rather than amino acid by amino acid. We have proposed that exposing the polypeptide chain in functional units contributes to fidelity in cotranslational processes such as targeting and folding (9). Depleting NAC from translating ribosomes led to at least two inappropriate interactions. First, the signal recognition particle (SRP), which interacts with signal peptides of nascent chains on ribosomes during targeting to the ER membrane, could be cross-linked to nascent chains lacking signal peptides (7). Second, in the absence of NAC ribosomes translating non-secretory polypeptides inappropriately interacted with translocation sites on ER membranes and the signal-less chains were even translocated, although with low efficiency. Adding back purified NAC to the system restored the fidelity of cotranslational ribosome binding and translocation of only secretory polypeptides (10). Recently the function of NAC in ribosome targeting to the ER was questioned (11, 12) in studies that used subphysiological concentrations of NAC. Although we were able to reproduce these results, we found that, when NAC was restored to physiological levels, our original observations were confirmed (1).

We have hypothesized that NAC normally prevents inappropriate interactions with nascent polypeptides and is thereby involved in transferring growing nascent chains to the appropriate cotranslationally acting factors. As a first step toward understanding how NAC might transfer control of the nascent chain to the proper factor, we asked whether specific functions could be assigned to each of the two polypeptides, which were always found in NAC preparations purified from bovine brain, and whether these two proteins are always found together as a complex. Here we report by using various techniques that highly purified NAC, as well as NAC in crude extracts, is a very stable complex composed of α and β subunits.

Human α- and βNAC were separately expressed in Escherichia coli and used to characterize the functions of the individual subunits and the reconstituted complex. Each subunit alone is in contact with the nascent chain on the ribosome. βNAC, but not αNAC, binds to ribosomes and prevents inappropriate ribosome binding to ER membranes. In contrast,
prevention of cross-linking of SRP to signal-less nascent chains requires the complete NAC complex. Although α-NAC alone is incapable of binding to ribosomes, it may contribute to NAC interaction with the ribosome or other cytosolic factors by virtue of its ability to bind tightly to nucleic acids, including those present in ribosomes and SRP.

**EXPERIMENTAL PROCEDURES**

**Chemical Cross-linking—** android pigmented body cells (100,000 x g supernatant in 20 mM Hepes/KOH pH 7.5, 150 mM KCl, and 1 mM DTT) was fractionated on a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) at 0.5 ml/min, and 0.5-ml fractions were collected. As was fractionated on a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) run with a gradient of 0.15 to 1.0 M KCl (in 20 mM Hepes, pH 7.5). On the following two columns excess, uncomplexed α-NAC was removed. Mono Q fractions over 400–470 mM KCl were combined, diluted to 150 mM salt, and applied to a Green A Matrex column (Amersham Pharmacia Biotech Biotech) with buffer supplemented with protease inhibitors and 0.8 unit/μl RNasin (Promega). RNACs were resuspended in ribosome binding buffer (RBB; 20 mM Hepes, pH 7.5, 100 mM KOAc, 5 mM Mg(OAc)2, 1 mM DTT, supplemented with protease inhibitors and 0.8 unit/μl RNAsin) using 0.5 volume of the buffer unit volume of the original translation mixture. Insoluble material was then removed by centrifugation at 14,000 rpm for 3 min at 4 °C. Recovery of the nascent chain complexes was typically 50–75%. These complexes were free of NAC as assessed by Western blotting (not shown) or by a site-specific photo-cross-linking approach.

**Cell Culture and Immunohistochemistry—**

**NAC Subunits Have Distinct Functions**

**In Vivo Transcription and Translation, and Isolation of Ribosome Nascent Chain Complexes—** In vivo transcription and translation of truncated mRNAs in rabbit reticulocyte lysates was done as described previously (16, 17). Transcribed mRNAs were translated for 20 min at 26 °C. After translation, 9 volumes of dilution buffer (40 mM Hepes, 0.5 M KOAc, 5 mM Mg(OAc)2, 2 mM DTT, pH 7.5) was added, and RNACs were visualized by Coomassie Brilliant Blue staining.

**Photo-cross-linking—**

**Preparation of Non-translating Ribosomes and Ribosomal Subunits—** Dog pancreas rough microsomes were prepared as described (21). Rough microsomes (in 250 mM sucrose, 50 mM Hepes-KOH, pH 7.6, 50 mM KOAc, 2 mM Mg(OAc)2, 1 mM DTT, 10 μg/ml polyethylthioglyoxal-benzoic acid (TDGA)-modified lys-rRNA was added to a reticulocyte lysate translation system, was done according to a previous study (19).

**Gel Retardation Assays (‘Band Shift Assays’)—** Incubation of DNA (0.4 μg of plasmid SK+ (Stratagene) digested with BanI) or RNA samples (1 μg of complete yeast tRNA (Boehringer), 2 μg of bovine ribosomal RNA (Sigma) in band shift buffer (10 mM Tris-HCl, pH 7.5, 150 mM KOAc, 15 mM Mg(OAc)2, 1 mM DTT). NAC was purified under denaturing conditions (presence of 6 M urea in all buffers) by the protease protection assay (Ref. 15, data not shown). NAC was also cloned into pET-28a (Novagen) and expressed in BL21(DE3) and refolded, and detergent was removed as described above. Following the same procedure as described for the GST fusion proteins, 6HisNAC could be reconstituted into a complex with RNAC, which was purified according to the same protocol as described above.

**NAC Subunits Have Distinct Functions**

**Cell Culture and Immunohistochemistry—**

MC3T3-E1 cells (clonal osteogenic cell line, (14)) were grown on glass coverslips to semi-confluence (25 kDa), and ribonuclease A (13.7 kDa).

**Preparation of Non-translating Ribosomes and Ribosomal Subunits—**

Dog pancreas rough microsomes were prepared as described (21). Rough microsomes (in 250 mM sucrose, 50 mM Hepes-KOH, pH 7.6, 50 mM KOAc, 2 mM Mg(OAc)2, 1 mM DTT, 10 μg/ml polyethylthioglyoxal-benzoic acid (TDGA)-modified lys-rRNA was added to a reticulocyte lysate translation system, was done according to a previous study (19).
NAC Subunits Have Distinct Functions

RESULTS

NAC Is a Complex of α and β Subunits—Purified NAC from bovine brain obtained after several chromatography steps consists of two polypeptides with apparent molecular masses of 33 and 22 kDa (7). To determine whether both polypeptides are required for NAC function we first asked whether they form a stable complex. Purified NAC migrated as a single moiety under non-denaturing conditions on PAGE (Fig. 1A, lane 1). When the band was excised and subjected to SDS-PAGE (under denaturing conditions) α- and βNAC were resolved as two bands (Fig. 1A, lane 2). Scanning and integrating the bands stained with Coomassie Brilliant Blue in lane 2 suggested a 1:1 stoichiometry. Other independent methods also support the hypothesis that NAC forms a heterodimeric complex of a 1:1 stoichiometry. Using the bi-functional, thiol-cleavable cross-linking reagent DSP (dithiobis(succinimidylpropionate), α- and βNAC were shown to form a complex, which was resolved by treatment with DTT (Fig. 1B). No homo-oligomers were detected with this method. Additionally, we subjected purified NAC to analysis by mass spectrometry (Fig. 1C). Three peaks were obtained that correspond to the predicted molecular masses of αNAC (23,492 Da), βNAC (17,352 Da), and the heterodimeric complex (40,864 Da), demonstrating that the differences in the calculated and apparent molecular masses deduced by SDS-PAGE are not the result of modifications but of an unusual running behavior in SDS-PAGE. The detection of a peak for the intact complex is an indication for a very stable complex.

Sizing chromatography reveals that in crude bovine brain extracts the NAC subunits are also in a complex (Fig. 1D). Note that no single subunits are detected in the appropriate fractions where the single subunits would be expected. NAC was also subjected to immunoprecipitation followed by Western blotting. In case of αNAC, all buffers and gradient solutions had to be supplemented with detergent (0.2% Triton X-100) and an inert protein (aprotinin, 20 μg/ml) to prevent aggregation of unbound βNAC. Centrifugation tubes were precoated with aprotinin to prevent unspecific binding of βNAC to the Ultraclear centrifugation tubes (Beckman).

Nascent Chain Targeting Assay (Floitation Assay)—Truncated RNCs in 0.5 volume of RBB (see above) were incubated with NAC or its individual subunits for 2 min at 26 °C and 5 min on ice prior to addition of 0.2 eq/μl EDTA/KOAc stripped rough microsomes (EKRM) (21). After 2 min at 26 °C and 5 min on ice, membranes and bound ribosomes were floated as described (23).

Fig. 1. NAC is composed of α and β subunits. A, gel electrophoresis of purified bovine NAC. Under non-denaturing electrophoresis α- and βNAC migrate together as a single moiety (lane 1). Upon a second electrophoresis under denaturing conditions, α- and βNAC are resolved as two bands (lane 2). B, chemical cross-linking of α- and βNAC. To purified NAC, the thiol-cleavable bifunctional cross-linker DSP (2 mM) was added and the proteins were fractionated by two-dimensional SDS-PAGE without dithiothreitol (DTT) in the first dimension and with DTT for the second dimension. L stands for loading sample of purified NAC. α- and βNAC heterodimers but no homodimers were detected. C, mass spectrometric analysis of purified bovine NAC using cytochrome c as a calibration standard (Cal). The three peaks obtained correspond to the estimated molecular masses of αNAC (23,492 Da), βNAC (17,352 Da), and the heterodimeric complex (40,864 Da), demonstrating that the differences in the calculated and apparent molecular masses deduced by SDS-PAGE are not the result of modifications but of an unusual running behavior in SDS-PAGE. The detection of a peak for the intact complex is an indication for a very stable complex.

NAC Is Localized in the Cytosol—NAC was originally purified from bovine brain cytosol and was associated with cytosolic ribosomes, suggesting that NAC is a cytosolic protein. Using a polyclonal anti-GST-αNAC serum (rather than affinity-purified antibodies) for immunofluorescence, it was recently claimed that αNAC is localized in the nucleus after serum starvation of MC3T3-E1 cells, a clonal osteogenic cell line (24). Therefore, we performed immunofluorescence microscopy using affinity-purified anti-α- and anti-βNAC peptide-specific antibodies to determine the localization of NAC in intact cells. Different cell lines (including COS-, HeLa-, Hep-G2, and HL60 cells) were tested for NAC localization by immunostaining. In all of these cases the vast majority of NAC was found in the cytoplasm (data not shown). Staining of MC3T3-E1 cells also suggested a cytosolic localization for both subunits regardless of whether the cells had been starved or not (Fig. 2). Cells were grown to semi-confluency. In parallel samples, cells were either arrested at the G0/G1 border by serum deprivation for 24 h (Fig.
the presence of fetal calf serum the vast majority of localization using peptide-specific affinity-purified antibodies. In the containing 10% fetal calf serum. Cells were fixed and analyzed for NAC in a parallel sample, the medium was exchanged against fresh medium for an additional 24 h or, cells at semi-confluency were either incubated with medium without the addition of fetal calf serum (“starvation”) for an additional 24 h or, because much higher yields could be achieved using recNAC or its individual subunits as indicated. Samples were irradiated and analyzed by SDS-PAGE and fluorography. Both subunits as well as the recombinant NAC could be cross-linked to nascent chains on the ribosome. Presence of the ribosome was essential, because puromycin treatment prior to irradiation abolished the cross-links (data not shown).

Reconstruction of Functional NAC from Individual Recombinant Subunits—To define the role of NAC subunits in the described biological functions, the two were separately expressed in E. coli and purified to homogeneity (Fig. 3, lanes 3 and 4). A 70-kDa protein often copurified during the initial steps with GST-αNAC, which was identified as DnaK (the Hsp70 homolog of E. coli) by Western blotting. Additionally, βNAC was expressed and purified as a his-tagged protein (Fig. 3, lane 4), because much higher yields could be achieved using this construct. Both βNAC variants (with or without his-tag) could be reconstituted into a functional αβ complex (see below). Recombinant reconstituted NAC (recNAC) could be purified to concentrations of 10–15 μM (Fig. 3, lane 2), which is 3- to 4-fold more concentrated than the highest achievable concentration of NAC purified from bovine brain (Fig. 3, lane 1).

Both Subunits of NAC Interact with Nascent Chains on the Ribosome—NACs contact to a nascent chain on the ribosome can be shown in a cross-linking approach where a photoreactivatable cross-linker is incorporated instead of lysine into the nascent chain. For the assay documented in Fig. 4 RNCs (ribosome nascent chain complexes) containing the amino-terminal 77 amino acids of peroxisomal firefly luciferase (77aaffLuc), which lacks an ER signal sequence, were prepared in a reticulocyte lysate system, supplemented with TDBA-modified lys-tRNA and [35S]methionine. RNCs were high salt-extracted and incubated with recombinant αNAC, his-tagged βNAC, recNAC, or NAC purified from bovine brain. After irradiation, cross-linking products were analyzed by SDS-PAGE followed by fluorography. The mass of the cross-linked protein can be estimated by deducting the mass of the nascent chain from that of the cross-linked product. In the absence of any added factors, no prominent cross-link appeared after irradiation (Fig. 4, lane 2). Both subunits, if added separately (Fig. 4, lanes 3 and 4), as well as in the reconstituted complex (Fig. 4, lane 5), are in close contact with the nascent chain, because they give rise to strong cross-links to the nascent chain. The cross-links are comparable in strength to those obtained with NAC purified from bovine brain (Fig. 4, lane 6), indicating that with regard to this function recNAC is as active as bovine NAC.

No cross-links to the NAC subunits were detected if the nascent chains were released from the ribosome by puromycin treatment prior to irradiation (data not shown). Although αNAC is clearly in close proximity to the nascent chain (a prerequisite for obtaining a cross-linking product with this method), its interaction with the RNC seems to be weaker than
that of βNAC. If the RNCs are sedimented through a low salt-containing (150 mM KOAc) sucrose cushion prior to irradiation, the cross-link to αNAC is hardly detectable, whereas the cross-link to βNAC remains unchanged (data not shown). This result implies that βNAC can bind to RNCs (see below) and αNAC, in the absence of the β subunit, is in rather loose contact with the nascent chain (see below).

αNAC Has a High Affinity for Nucleic Acids—αNAC acts at the ribosome where it is in close vicinity not only to various cytosolic and ribosomal proteins but also to several different RNA molecules, e.g. ribosomal RNA, mRNA, tRNA, and 7SL RNA of the ribonucleoprotein complex SRP. Therefore, we were interested in the question whether αNAC, which does not bind to non-translating ribosomes (see below), can bind to nucleic acids. Interestingly, it had been recently reported that αNAC binds to DNA in a sequence-specific manner. A consensus sequence for binding of αNAC to DNA was published (5'-G/C)A(G/C)(G/C)ANNNG-3' (25). We confirmed binding of αNAC to oligonucleotides containing this motif; however, we also observed binding to oligonucleotides where this motif was mutated (data not shown). To further characterize the binding of αNAC to nucleic acids, we tested binding to DNA, rRNA, tRNA, and 7SL RNA. DNA binding was tested using the plasmid pBluescript SK+, which was cleaved with BanI, resulting in four easily distinguishable fragments (244, 386, 1097, and 1231 bp). Only one contains the so-called “NAC motif” (1097 bp). The DNA fragments were incubated with increasing amounts of αNAC and separated on agarose gels containing ethidium bromide. All four DNA fragments exhibit a slower mobility in the gel, indicating that αNAC binds to them regardless of whether they contained the NAC motif or not (Fig. 5a). Furthermore, the more αNAC is added, the stronger the retardation effect becomes, indicating that more than one αNAC molecule binds to each of the DNA fragments.

αNAC binding is not restricted to DNA molecules. Incubation of ribosomal RNA from bovine liver (Fig. 5b) or complete tRNA from yeast (Fig. 5c) with rising amounts of αNAC also resulted in a complete shift of the RNA band(s). αNAC also bound to the 7SL RNA in an almost 1:1 stoichiometry (data not shown). In summary, αNAC binds to all nucleic acids we have tested. Clearly, this affinity is not restricted to DNA molecules and is not dependent on a specific sequence motif. The ability of αNAC to bind to RNA and DNA molecules might contribute to the overall binding of the NAC complex to ribosomes or may even underlie its ability to compete with SRP for access to nascent chains (see below). If this capacity contributes to the overall binding of the complex to translating ribosomes, RNA molecules should compete for the interaction of αNAC with the nascent chain. A 10-fold molar excess of mRNA (coding either for the first 77 amino acids of firefly luciferase or the first 86 amino acids of preprolactin), tRNA, or rRNA led to a 50% reduction of the cross-link of αNAC to 77aIfLuc RNCs, whereas cross-links to the NAC complex remained unchanged in the presence of RNAs (data not shown).

βNAC, but Not αNAC, Can Bind to the Ribosome in the Absence of the Corresponding Subunit—βNAC can be sedimented with RNCs and prevents RNCs as well as non-translating ribosomes from binding to translocation sites at the ER membrane by blocking a putative ribosomal membrane attachment site (26). We investigated whether the affinity for ribosomes could be ascribed to one subunit or whether this binding requires NAC complex. recNAC or the individual subunits were incubated with non-translating cytosolic ribosomes or with the large 60 S ribosomal subunit. Samples were sedimented through a low salt sucrose cushion. Each tube was divided into four fractions (top (T), middle (M), bottom (B), and pellet (P)). Fig. 6, shows the distribution of NAC subunits in these fractions after SDS-PAGE and Western blotting. Bottom and pellet fractions contained sedimented ribosomes as judged by Ponceau and Coomassie staining (data not shown). In the absence of ribosomes, none of the NAC proteins sedimented (Fig. 6, -ribos). The reconstituted, recombinant complex bound to non-translating ribosomes and, although less efficiently, to 60 S ribosomal subunits (Fig. 6, recNAC, B and P). αNAC alone is not capable of binding to non-translating ribosomes or 60 S ribosomal subunits. It was always found in the top fraction. βNAC showed the same binding pattern as recNAC. It bound to 80 S ribosomes and less efficiently to 60 S subunits.

βNAC Alone Prevents Default Targeting of Ribosomes Containing Signal-less Nascent Chains to the ER—In addition to its role in shielding the nascent chain on the ribosome toward the cytosol, NAC prevents inappropriate targeting of non-translating ribosomes and RNCs bearing signal-less nascent chains to the ER membrane by occupying the proposed membrane-attachment site (M-site) on the ribosome (23, 26). We next investigated whether the binding of βNAC and recNAC to ribosomes observed in Fig. 6 reflects functional binding to the M-site on the ribosome. This function is tested by using a flotation assay where high salt-stripped RNCs are incubated with microsomal membranes (EKRM), which contain the com-

**FIG. 5. αNAC binds to nucleic acids.** a, pBluescript SK+ was digested with BanI resulting in four easily distinguishable fragments prior to incubation with increasing amounts of αNAC (0.4, 0.8, 1.7, and 2.5 μM). Samples were separated on agarose gels, and DNA was visualized by ethidium bromide staining. Note that all four fragments exhibit a slower mobility in the gel due to binding of αNAC, although only one of them (1097 bp) contains the so-called NAC motif (asterisk). b, ribosomal RNA from bovine liver (Sigma, lane 1) was incubated with BSA (25 μg/ml, lane 2) and increasing amounts of αNAC (0.5 μM, lane 3; 1.5 μM, lane 4; and 3 μM, lane 5). Samples were separated on an agarose gel, and RNA was visualized by ethidium bromide staining. αNAC (lanes 3–5), but not the unspecific protein (BSA, lane 2), bound to ribosomal RNA as indicated by the change of mobility in the gel, c, complete tRNA from yeast (Sigma, lane 1) was incubated with BSA (25 μg/ml, lane 2) and increasing amounts of αNAC (0.5 μM, lane 3; 1.5 μM, lane 4; and 3 μM, lane 5). Samples were separated on an agarose gel, and RNA was visualized by ethidium bromide staining. The addition of an unspecific protein (BSA, lane 2) did not change the mobility of the tRNA in the gel (compare lanes 1 and 2). Addition of αNAC (lanes 3–5) led to a band shift indicating the binding of this subunit to tRNA.
NAC Subunits Have Distinct Functions

**Fig. 6.** Binding of NAC proteins to non-translating ribosomes. Recombinant NAC (0.6 μM) or its individual subunits (0.7 μM, each) were incubated with dog pancreas 80 S ribosomes (0.15 μM) or 60 S ribosomal subunits (0.15 μM) or in the absence of ribosomes. Ribosomes were sedimented through low salt sucrose cushions, which were divided into top (T), middle (M), and bottom (B) fractions, and pellets (P) were resuspended using 1% SDS. Ribosomes are recovered under these conditions in the bottom and pellet fraction. NAC was detected in the different fractions by Western blotting with peptide-specific antibodies against α- and βNAC. In the absence of ribosomes or 60 S ribosomal subunits, NAC and its subunits did not sediment into the sucrose gradient (lanes 9–12, -ribos), they were always recovered in the top fraction. αNAC did not bind to 80 S ribosomes or 60 S subunits. It was always recovered in the top fraction (lanes 1 and 5). In contrast, recombinant NAC as well as βNAC alone bound to complete 80 S ribosomes and 60 S ribosomal subunits (lanes 3, 4, and 8). Note that the binding to 60 S ribosomal subunits appears to be weaker than to 80 S ribosomes.

**Fig. 7.** βNAC prevents binding of RNCs to ERKMs. High salt-stripped 77aaffLuc RNCs prepared in rabbit reticulocyte lysate with TDBA-lys-tRNA and [35S]methionine were incubated with bovine NAC, recombinant NAC or its individual subunits as indicated prior to addition of 2 eq of ERKMs. Samples were subjected to the flotation assay and then divided into top fractions (T) containing membrane-bound RNCs and bottom fractions (B) containing free RNCs. In the absence of NAC or its subunits, approximately 50% of the RNCs bound to membranes (lanes 1 and 2). Addition of bovine NAC (2 μM, lanes 3 and 4), recombinant NAC (1.7 μM, lanes 9 and 10), or the β subunit alone (1.1 μM, lanes 7 and 8) prevented this binding. In contrast, addition of αNAC (2 μM, lanes 5 and 6) had no influence on RNC binding to membranes.

**DISCUSSION**

Between synthesis on the ribosome and arrival at a final destination, cellular proteins undergo a journey whose regulation and control is vital to the proper functioning of the eukaryotic cell (27). Thus, depending on their final localization, proteins may interact with the cytosolic folding machinery, i.e. chaperones (28, 29), or with factors like SRP and SRP receptor, which directs cotranslational translocation into the ER (30–33). Although the mechanisms by which proteins are translocated into the ER are very well understood, our knowledge about other cotranslationally acting factors such as cytosolic chaperones and nascent polypeptide-associated complex (NAC) is still rudimentary.

NAC is the first cytosolic factor to contact the nascent polypeptide chain as it grows on the ribosome and thus may be key in regulating early events in protein folding and transport (8). In fact, it has been established that NAC prevents SRP binding to inappropriate nascent chains, i.e. those lacking a signal peptide (7). NAC also prevents binding of inappropriate ribosomes to translocons on the ER by blocking the putative ribosomal M-site (1, 10, 23, 26). We have previously hypothesized that, as the nascent chain lengthens, NAC binding weakens, and for signal sequence containing proteins, allows SRP to compete effectively for binding to signal peptides. Once SRP displaces NAC, the ribosome is targeted to the ER, and after the SRP receptor displaces the SRP, the M-site is free to bind to the translocon and the nascent chain enters the lumen of the ER (23).

The physiological importance of NAC cellular function is underscored by the embryonically lethal phenotype of NAC deletion mutants in both mouse (2) and fruit fly (3). An insertional mutation in the gene coding for βNAC led to early postimplantational lethality in mice (2). In Drosophila a mutation affecting βNAC expression was shown to cause the long known bicaudal phenotype (3). Bicaudal was the first Drosoph-
NAC Subunits Have Distinct Functions

ilar mutation identified as producing mirror-image pattern duplications along the anterior-posterior axis of the embryo (35). In contrast, deletion mutants in yeast, lacking both αNAC and βNAC, show only slight growth defects (4, 5), but SRP is also not essential in yeast (35, 37).

We show here that NAC is detected solely as a heterodimer of α and β subunits associated in a very stable complex in vivo (Fig. 1). Consistent with this, peptide-specific antibodies raised against the individual subunits demonstrate that both have a cytoplasmic localization in a number of cell lines, including HepG2, HeLa, and COS and during different cell cycle stages of HL60 cells.2 This is also true in an osteogenic cell line, MC3T3-E1 after serum starvation (Fig. 2), conditions previously reported to result in movement of αNAC to the nucleus (24). Because of NAC’s extremely high intracellular concentration of 3 to 10 μM in tissues tested (1), it is impossible to exclude that a minor fraction of NAC might be in the nucleus but, clearly, NAC is not depleted from the cytosol in favor of a nuclear localization under these conditions.

We present here a further characterization of NAC based on recombinant subunits purified from E. coli (Fig. 3). Full activity in all of our assays was obtained using holoNAC reconstituted from these recombinant subunits (Figs. 4, 7, and 8). This confirms that NAC is composed solely of α and β subunits as well as showing that our recombinant subunits are intact and functional.

Cross-linking experiments demonstrate that both α and β subunits are in contact with nascent chains on the ribosome (7). However, βNAC binds more tightly than αNAC, because its binding is resistant to low salt extraction and it retains binding to non-translating ribosomes (Fig. 6). In fact, βNAC binding to ribosomes is sufficient to prevent the mistargeting of signalless RNAs to the ER (Fig. 7). This suggests that βNAC binds at or near the proposed M-site on the ribosome, preventing binding to the translocon.

The only activity that we can so far attribute to αNAC on its own is the ability to bind nucleic acids (Fig. 5). Yotov and St-Arnaud have also published that αNAC binds to a highly redundant consensus DNA sequence and thereby is implicated in transcription activation (25). Consistent with this idea, it has been reported that a tissue-specific isoform of αNAC (skNAC) is up-regulated in differentiating myoblasts and osteoblasts and in cells involved in wound healing (24, 25, 39, 40). Note, however, that all biochemical activities reported up to now have been for αNAC and not skNAC. In contrast to Yotov and St-Arnaud, we find that αNAC binding is not restricted to DNA, nor is it sequence specific. Rather, αNAC binds to a variety of DNA and RNA molecules, including ribosomal RNA, tRNA (Fig. 5), and the 7SL RNA of SRP.3 These results suggest that αNAC affinity for nucleic acid may contribute to the overall binding and possibly positioning of NAC in complex with the ribosome. This hypothesis is supported by the observation that RNase A treatment of ribosomes results in less holoNAC binding to ribosomes.3 Interestingly, evidence has recently been provided that 28 S RNA mediates the interaction of the ribosome with the SEC61 complex (38), which together with NAC and SRP, has been shown to compete for binding to the M-site (23). Also consistent with this idea is our unpublished finding that both tRNA and mRNA compete for the cross-link of αNAC to the nascent chain but have no effect on the cross-link of holoNAC to nascent chains. However, ribosomal RNA may only become accessible to αNAC during translation or in the presence of βNAC, because αNAC alone is not capable of binding to 80 S ribosomes. A computer model constructed with Predict-Protein software (41) assigns the sequence of αNAC from Arg-71 to Lys-82 to an α-helix. Because this putative α-helix would carry a substantial positive charge, it may be responsible for a strong but nonspecific interaction with the negatively charged phosphates of nucleic acids.

At least one key function of NAC requires both α and β subunits in complex. HoloNAC is required to prevent inappropriate interactions of SRP with non-signal sequence nascent chain complexes (Fig. 8). Thus, although βNAC is able to bind the ribosome on its own, α and β subunits together bind in a functionally different manner.

These data together suggest a model in which NAC is bound to the ribosome via its β subunit binding to the M-site. βNAC, by complexing tightly to αNAC also serves to strengthen αNACs interaction with the ribosome. Together, the subunits shield the nascent chain against early inappropriate interactions with other factors. We suggest that cycles of binding and releasing of NAC gradually allow other cytosolic factors to access the growing polypeptide, in “quantal” units, rather than one amino acid at a time. A question to be addressed in the future is whether NAC interacts with any of the protein factors that replace it on the nascent chain. For example, αNAC contains a domain with homology to DnaJ and GST-αNAC copurifies with DnaK, the Hsp70 homolog in E. coli,4 suggesting that αNAC interacts with Hsp70 in eukaryotic cells. Our purified recombinant α- and βNAC subunits constitute a powerful tool to answer such questions and to further characterize the M-site.

Acknowledgments—We thank Drs. T. Mayer and D. H. Smith for critical comments and help in preparing the manuscript.

REFERENCES
1. Möller, I., Beatrix, B., Krebsich, G., Sakai, H., Lauring, B., and Wiedmann, M. (1998) FEBS Lett. 441, 1–5.
2. Deng, J. M., and Behringer, R. R. (1995) Transgenic Res. 4, 264–269.
3. Markesich, D. C., Gajewski, K. M., Nazimiee, M. E., and Beckingham, K. (2000) Development 127, 559–572.
4. Reinman, B., Bradsher, J., Franke, J., Hartmann, E., Wiedmann, M., Prehn,
5. Beatrix, A. Koff, and M. Wiedmann, unpublished results.
6. Beatrix and M. Wiedmann, unpublished observations.
NAC Subunits Have Distinct Functions

...
