

NAC Covers Ribosome-associated Nascent Chains Thereby Forming a Protective Environment for Regions of Nascent Chains Just Emerging from the Peptidyl Transferase Center

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Abstract. We demonstrate that nascent polypeptide-associated complex (NAC) is one of the first cytosolic factors that newly synthesized nascent chains encounter. When NAC is present, nascent chains are segregated from the cytosol until ~30 amino acids in length, a finding consistent with the well-documented protease resistance of short ribosome-associated nascent chains. When NAC is removed, the normally protected nascent

chains are susceptible to proteolysis. Therefore NAC, by covering COOH-terminal segments of nascent chains on the ribosome, perhaps together with ribosomal proteins, forms a protective environment for regions of nascent chains just emerging from the peptidyl transferase center. Since NAC is not a core ribosomal protein, the emergence of nascent chains from the ribosome may be more dynamic than previously thought.

THE structure of the ribosome and the mechanisms and factors involved in initiation, elongation, and termination of translation have been extensively studied (for reviews see: Lake, 1985; Kozak, 1992; Merrick, 1992; Nierhaus, 1993; Noller, 1993; Tuite and Stansfield, 1994). Other events that occur at the ribosome after peptide bond formation are less well characterized. There are several critical cellular functions that can occur cotranslationally, such as targeting to organelles (Pfanner et al., 1992; Rapoport, 1992; Gilmore, 1993; Wickner, 1994), folding of proteins to achieve their active conformations (Hartl et al., 1994), and various enzymatic modifications (NH₂-terminal acetylation [Strous et al., 1974], de-formylation of the first formyl-methionine [Adams, 1968], cleavage of initiator methionyl residues [Jackson and Hunt, 1970], hydroxylation of proline and lysine residues [Lazarides et al., 1971], disulfide bond formation [Bergman and Kuehl, 1979], N-glycosylation [Glabe et al., 1980], and incorporation of heme into globin [Komar et al., 1993], etc.). Understanding how and where nascent chains emerge from ribosomes is critical to understanding at least those events that take place in the cytosol, and possibly beyond that in the ER. Although previous experiments designed to determine how nascent chains become exposed to the cytosol have produced contradictory results, it is

generally accepted that newly synthesized polypeptide sequences pass through a putative tunnel inside the large ribosomal subunit, the length of which is ~35-40 amino acids, before being exposed to the cytosol. Evidence for this model comes largely from protease protection experiments (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Bernabeu and Lake, 1982; Bernabeu et al., 1983; Milligan and Unwin, 1986; Yonath et al., 1987). In contrast, Ryabova et al. (1988) contend that no such tunnel exists and that nascent chains are almost immediately located in the cytosol after synthesis.

Although both models seek to explain where chains are exposed (either at the far end of the tunnel, opposite the peptidyl transferase center [PTC¹—located at the bottom of the central protuberance on the large ribosome subunit] or at the site of the PTC itself), neither model addresses the question of how proteins are released in a manner that ensures the proper cotranslational interaction of nascent chains with cytosolic nascent chain binding factors. No matter where the exit site is actually located, both models envision that polypeptides emerge from the ribosome one amino acid at a time. The following example illustrates why this is potentially problematic. Proteins destined for translocation across the ER membrane are synthesized with an amino-terminal signal peptide that when fully exposed is bound by the signal recognition particle (SRP) (Ng and Walter, 1994). Partially exposed signal peptides,

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1. *Abbreviations used in this paper:* NAC, nascent polypeptide-associated complex; PTC, peptidyl transferase center; SRP, signal recognition particle; TBB, translation blank buffer; CTABr, cetyltrimethylammonium bromide.

which are not yet exposed enough to be bound by SRP, contain hydrophobic cores that should be good substrates for cytosolic chaperones (Hartl et al., 1994). More generally, how does the cell prevent incorrect or inappropriate interactions from occurring at the ribosomal exit site?

Another potential problem with the proposed rigid tunnel model is that some polypeptide sequences, by chance, might have affinity for the walls of the tunnel and therefore get "stuck" inside the tunnel.

Nascent polypeptide-associated complex (NAC) has been identified as a cytosolic factor that associates exclusively to ribosome-associated nascent, as opposed to released, polypeptide chains (Wiedmann et al., 1994). Importantly, because NAC can interact with regions of nascent chains as close as 35 amino acids from the PTC, we imagine that NAC functions at the interface of the ribosome and cytosol. Using a novel controlled protease digestion (factor Xa) assay and an established photocross-linking approach (Görlich et al., 1991), we have determined that NAC protects short nascent chains and cross-links to regions of nascent chains as close as 17 amino acids to the PTC and is therefore likely to be one of the first cytosolic factors that nascent chains encounter. As hypothesized (Neupert and Lill, 1994), we show that when bound, NAC prevents interaction of nascent chains with other cytosolic factors until it is released when the chains are sufficiently elongated. Because a ribosomal tunnel for nascent chains was defined by protease protection data and because NAC protects nascent chains, we conclude that nascent chains do not traverse a long rigid tunnel composed of core ribosomal proteins but are instead covered by cytosolic proteins, including NAC, probably on the surface of the ribosome. In this way, the presence of NAC gives the appearance of a tunnel.

Materials and Methods

Plasmid Construction

Plasmid pGEM-luc (Promega Corp., Madison, WI) contains the firefly luciferase gene under the control of a SP6 promoter. It was used to construct a set of plasmids which, after *in vitro* transcription/translation, give rise to polypeptides containing a factor Xa recognition site (Nagai and Thøgersen, 1984). The plasmid pGEM-luc was digested with *Nar*I and *Eco*RV and a linker was introduced into the digested pGEM-luc DNA (top strand: 5' CG CCA AAC ATG ACC ATC GAA GGT AGA ATG GGA T 3'; bottom strand: 5' A TCC CAT TCT ACC TTC GAT GGT CAT GTT TGG 3') to generate the plasmid SM-1. The first 13 amino acids of the NH₂-terminal polypeptide encoded by the plasmid SM-1 are identical to those of firefly luciferase. This is followed by a sequence dictated by the linker sequence. The underlined nucleotides in the top strand encode the four amino acid factor Xa recognition sequence, Ile-Glu-Gly-Arg, positioned 20 amino acids from the NH₂ terminus. SM-2 and SM-3 are derivatives of SM-1. SM-1 was digested with *Cla*I and *Xho*I to remove 344 nucleotides. Blunt ends were produced on the remaining DNA fragment using Klenow enzyme. The plasmid pBluescript SK⁻ (Stratagene Inc., La Jolla, CA) was digested with *Bst*XI and *Xho*I to excise the polylinker region that was isolated and blunt-ended with Klenow enzyme and T4 DNA polymerase. Ligation was carried out between the polylinker fragment of pBluescript SK⁻ and SM-1. The pBluescript SK⁻ *Bst*XI/*Xho*I fragment was ligated into SM-1 *Cla*I/*Xho*I in both orientations. In SM-2 the *Bst*XI site of the polylinker was fused with the *Cla*I site of SM-1. In SM-3 the *Xho*I site of the polylinker was fused to the *Cla*I site of SM-1. SM-4 was derived from SM-3 by ligating a linker (top strand: 5' CTA GTG ATC AGC CTC ATG AAG ATC TTC GAA TAT TTA AAG AGC T 3'; bottom strand: 5' CTT TAA ATA TTC GAA GAT CTT CAT GAG GCT GAT CA 3') into the *Spe*I and *Sac*I digested SM-3 plasmid.

Transcription, Translation, and Cross-linking

In vitro transcription of linearized plasmids was done according to Gilmore et al. (1991). *In vitro* translation was done either in the reticulocyte lysate system (Jackson and Hunt, 1983) or in the wheat germ system (Ericson and Blobel, 1983). If not otherwise indicated, translation reactions were incubated for 20 min at 26°C in the presence of 1 mCi/ml [³⁵S]methionine (specific activity 1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL). Photocross-linking using TDBA-lys-tRNA was described in detail by Görlich et al. (1991).

Isolation of Nascent Chain-Ribosome Complexes

For the high salt isolation of the nascent chain-ribosome complexes, 10 vol of ice-cold, high salt dilution buffer (HSDB: 500 mM KOAc, 50 mM Hepes/KOH, pH 7.5, 5 mM Mg(OAc)₂, 1.5 mM DTT, 0.4 U/μl of placental RNase inhibitor, and 0.5 mM cycloheximide) was added. After 10 min on ice, samples were layered onto sucrose cushions and centrifuged (0.5 M sucrose in HSDB; Beckman rotor TLA 120.1 for 20 min at 120K rpm). The ratio of the sucrose cushion volume to the sample volume was ~2:1. Low salt isolation was as for high salt, with the exception that KOAc was at 100 mM and samples were not diluted before sedimentation. Isolated nascent chain-ribosome complexes were then resuspended in translation blank buffer (TBB: 50 mM Hepes/KOH pH 7.5, 100 mM KOAc, 5 mM Mg(OAc)₂, 2 mM DTT, 0.028 mM each amino acid, 0.25 mM GTP, 1.25 mM ATP, 8 mM creatine phosphate, 0.075 mg/ml creatine kinase, and 0.8 U/μl placental RNase inhibitor) in their original volume.

Factor Xa Protease Digestion

Blood coagulation factor Xa protease (NEB) was added directly to the translation assay after translation was stopped with cycloheximide or to isolated nascent chain-ribosome complexes. 1 μl factor Xa (1 μg) was added to 10 μl sample. Digestion was carried out for 20 min on ice. The factor Xa stock solution contained 20 mM Hepes, pH 8.0, 500 mM NaCl, 2 mM CaCl₂, and 50% glycerol. The reaction was stopped by adding PMSF to 2 mM.

Cetyltrimethylammonium Bromide (CTABr) Precipitation of Nascent Chains

To a 12 μl reaction, 250 μl of a 2% CTABr solution was added and the samples were mixed. Then 250 μl of 0.5 M NaOAc, pH 5.4 was added together with 50 μg yeast tRNA. After incubation on ice for 10 min, samples were centrifuged for 10 min at 14,000 rpm (Eppendorf centrifuge, 4°C). Pellets were extensively washed with acetone:HCl (19:1) to remove CTABr and then were resuspended in SDS-PAGE sample buffer (Gilmore et al., 1991).

Elongation of Nascent Chains After Isolation through Sucrose Cushion Centrifugation

Transcripts were translated *in vitro* for 1 min at 26°C in the wheat germ or reticulocyte lysate system as described above. Translation was arrested by incubation on ice and the nascent chain-ribosome complexes were high salt extracted and isolated through high salt sucrose cushions by centrifugation. The complexes were resuspended in TBB and the complete translation mixture including excess nonradioactive methionine was added. Samples were then incubated for 20 min at 26°C.

In a similar experiment involving elongation of nascent chains after cross-linking to NAC, the transcripts were translated in the presence of TDBA-lys-tRNA for 1 min. Nascent chain ribosome complexes were isolated under low salt conditions and then irradiated after resuspension in TBB but before further elongation. Samples were then elongated as above. After elongation, the samples were fractionated by a second low salt sucrose centrifugation step as described.

Protection of Nascent Chains from Factor Xa Cleavage

Transcripts for 32aaSM-1 or 44aaSM-2 were translated in the wheat germ system, treated with high salt and the nascent chain-ribosome complexes were isolated through a high salt sucrose cushion as described. After resuspension in TBB, wheat germ cytosol (35–50 mg/ml), bovine brain cytosol (~30 mg/ml), NAC depleted bovine brain cytosol (~30 mg/ml), or purified NAC (2 μM in NAC buffer [330 mM KCl, 20 mM Hepes, pH 7.5,

and 25% glycerol) was added. Samples were then incubated first for 10 min at room temperature and then for 20 min on ice. Factor Xa was added to the samples as indicated and digestion was carried out on ice for 20 min. Puromycin was added to lanes, as indicated in Fig. 3, at a concentration of 1 mM and incubated for 10 min at 37°C. RNase A was routinely added to the samples treated with puromycin at a concentration of 1 mg/ml and samples were incubated at 37°C for 10 min.

Immunoprecipitation and Product Analysis

NAC cross-link adducts were treated with RNase A (100 µg/ml) for 10 min at 30°C to digest peptidyl-tRNAs. The samples were adjusted to 1% SDS, boiled, diluted 10-fold, and immunoprecipitated by a standard procedure (Harlow and Lane, 1988). α- and βNAC antibodies were raised in rabbits against synthetic peptides corresponding to the COOH-terminal sequence of human αNAC (These sequence data are available from EMBL/Genbank/DBJ under accession number X80909.), RALKNN-SNDIVNAIMELTM-COOH, and human βNAC (BTF3b) (These sequence data are available from EMBL/Genbank/DBJ under accession number X53281.), ENFDEASKNEAN-COOH, respectively.

Proteins were separated on 15% acrylamide/0.6% bisacrylamide SDS-gels (Laemmli, 1970) or on 15.5% acrylamide/1% bisacrylamide tricine gels. (Schägger and von Jagow, 1987). Reticulocyte translation mixtures were precipitated with ammonium sulfate to remove bulk globin (Nischitta and Blobel, 1989). After PAGE, gels were first washed for 5 min in 40% methanol/10% acetic acid, 5 min in Enlightening (DuPont NEN, Boston, MA) and then dried. Exposure of the gels was at -80°C on Kodak X-OMAT film.

Results

Experimental Strategy

To investigate the environment of ribosome-associated nascent chains, a homogeneous population of nascent chains of defined length and sequence were synthesized by *in vitro* translation of truncated mRNAs lacking stop codons. After translation, these chains remain stably associated with ribosomes as peptidyl-tRNAs (Gilmore et al., 1991). In combination with the truncated mRNA technique, two approaches have been used to probe the environment of nascent chains. In one approach we have used a site-specific protease, added to the cytosol, to ask when and under which conditions specific regions of emerging nascent chains are accessible to proteinaceous cytosolic probes. More specifically, we have introduced a site-specific factor Xa protease cleavage site at defined positions in nascent chains. Factor Xa is a sequence-specific protease, recognizing a four-amino acid sequence Ile-Glu-Gly-Arg and cleaving after arginine (Nagai and Thøgersen, 1984).

In a second approach we examined the system from the perspective of the nascent chain by asking which molecules are in contact with the chain during synthesis. This was accomplished by cotranslationally incorporating photo-activatable cross-linking probes (Brunner, 1993) at defined positions in nascent chains. In this established technique the ε-amino group in the lysyl-tRNA is modified with the succinimido ester of 4-(3-trifluoromethyl-diazirino) benzoic acid to produce TDBA-lys-tRNA (Görlich et al., 1991). When added to *in vitro* translation reactions, TDBA-lys is incorporated into polypeptides as dictated by the position of lysine codons in the mRNA. Upon photoactivation, a carbene capable of inserting into quasi-inert C-H bonds is generated. As carbenes have an extremely short half-life and high reactivity (Brunner, 1993), obtaining a cross-link indicates that the cross-linked

proteins are in direct contact with these residues in the nascent chain.

We used these two techniques in parallel to determine when a nascent chain emerges from the ribosome and is exposed to the cytosol. If nascent chains traverse a rigid tunnel comprised of core ribosomal proteins (defined as those that resist salt extraction from the ribosome, Kurland, 1971; Staehelin and Falvey, 1971), then high salt extraction should neither alter the spectrum of proteins cross-linked to the regions of the nascent chain in the tunnel nor influence the protease resistance of these same regions. In contrast, if salt extractable cytosolic factors cover those regions of the nascent chain, then salt extraction should alter the cross-linking pattern and eliminate protease protection.

Constructs for translation of truncated mRNAs were made in which factor Xa recognition sites were introduced 20 amino acids from the amino termini (Fig. 1). A multi-cloning site was introduced carboxy-terminal to the engineered factor Xa cleavage site in both orientations. This allows for production of mRNAs encoding two different protein sequences distal to the factor Xa cleavage site and for the availability of many restriction enzyme sites for synthesis of truncated mRNAs of virtually any length (Fig. 1, SM-2 and SM-3). Derived from SM-3, SM-4 has additional restriction enzymes sites. Since these constructs were used in parallel for photocross-linking experiments, attention was given to the lysine positions as well.

Factor Xa Cleavage of Nascent Chains

Fig. 2 A demonstrates that factor Xa can cleave long nascent and terminated polypeptides after *in vitro* translation. 116aaSM-1 is a truncated mRNA lacking a stop codon that encodes a 116-amino acid long nascent chain containing a factor Xa recognition site 20 amino acids from the amino terminus. This chain remains ribosome associated after *in vitro* translation. In contrast, 126aaSM-1

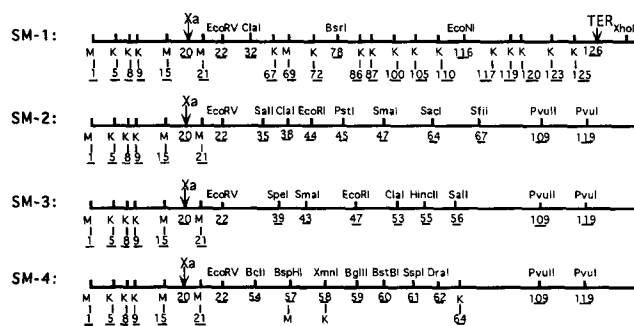


Figure 1. SM constructs. A factor Xa recognition sequence (Ile-Glu-Gly-Arg) was introduced 20 amino acids from the NH₂ terminus into the firefly luciferase gene encoded by the plasmid pGEM-luc (for details see Materials and Methods). The plasmid SM-1, where lysines are present throughout the polypeptide, was used to construct the plasmids SM-2 and SM-3. In these latter two constructs lysines are located exclusively near the NH₂ termini. SM-2 and SM-3 were created by inserting a blunt-ended polylinker into SM-1 in both orientations, so that they encode different amino acid sequences downstream of the insertion site. Derived from SM-3, SM-4 has several more restriction sites. Xa, activated human blood-coagulation factor X; M, methionine; K, lysine.

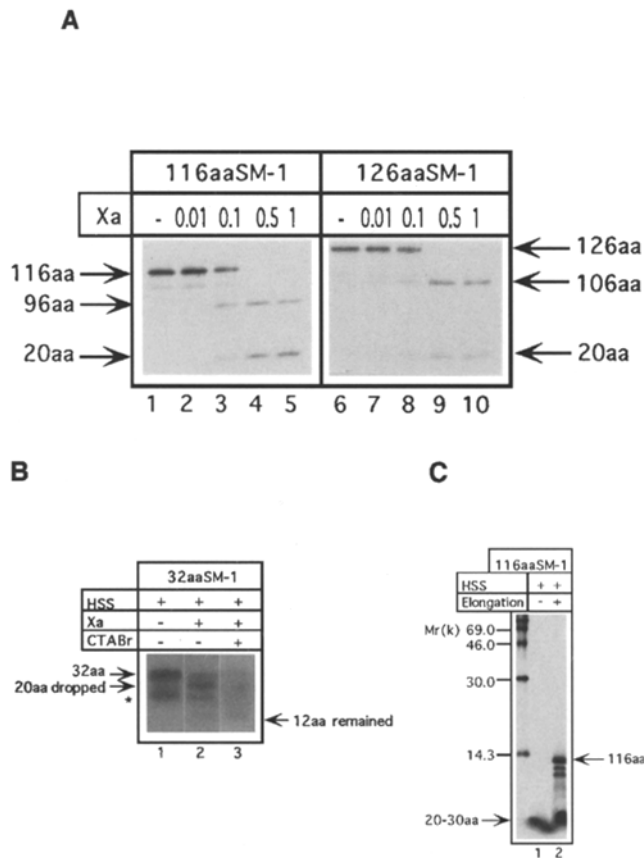


Figure 2. Factor Xa cleavage of nascent chains. (A) 116aaSM-1 or 126aaSM-1 was translated in the wheat germ lysate system with [³⁵S]methionine. Translation assays were gel-filtrated through a Sephadex G-50 column equilibrated in TBB to remove the unincorporated [³⁵S]methionine. Samples were divided, and factor Xa was titrated at 0, 0.01, 0.1, 0.5, and 1 μg per 10-μl sample. Digestion was carried out on ice for 20 min and stopped by addition of PMSF. Samples were not TCA precipitated because the small peptides generated by factor Xa might not precipitate. Proteins were separated on a 15.5% acrylamide tricine gel and visualized by fluorography. The 20-amino acid peptide is recovered with low efficiency because the fixation and enhancing procedures extract some of the peptides from the gel. (B) After in vitro translation of 32aaSM-1 in the wheat germ lysate system supplemented with [³⁵S]methionine, nascent chain-ribosome complexes were extracted with high salt and isolated by centrifugation through a high salt sucrose cushion. After resuspension in TBB, a portion of the sample was kept on ice as a control (lane 1). The remaining sample was subjected to factor Xa digestion. Samples were either directly applied to a 15.5% acrylamide tricine gel (lane 2) or first precipitated by 2% CTABr and then subjected to electrophoresis (lane 3). The asterisk shows the nascent chain produced by the second ribosome that initiated on the truncated mRNA. Again, small peptides are underrecovered. (C) 116aaSM-1 was translated in the wheat germ lysate system supplemented with [³⁵S]methionine for 1 min at 26°C. The translation reaction was placed immediately on ice to stop elongation. The nascent chain-ribosome complexes containing very short nascent chains were treated with high salt and isolated by centrifugation through a high salt sucrose cushion. After resuspension in TBB, a complete wheat germ translation mixture containing excess nonradioactive methionine was added to the isolated nascent chain-ribosome complexes. One-half of the sample was kept on ice as a control (lane 1). The other half was incubated at 26°C for 15 min to allow the stalled ribosomes to further elongate the previously initiated

is a similar construct but contains a stop codon. The polypeptide encoded by 126aaSM-1 is released from the ribosome after synthesis. Increasing amounts of factor Xa resulted in more cleavage of both constructs. Cleavage of 116aaSM-1 polypeptide generated two fragments, a 20 amino acid fragment and a 96-amino acid ribosome-associated fragment (Fig. 2 A, lanes 1-5). Cleavage of 126aaSM-1 polypeptide generated two fragments of 20 and 106 amino acids (Fig. 2 A, lanes 6-10). When factor Xa was applied to nascent chains without the protease recognition sequence, e.g., firefly luciferase nascent chains, cleavage was not observed (data not shown).

Because the factor Xa cleavage site in the 116aaSM-1 was far away from the PTC, cleavage was expected. We next sought to determine whether factor Xa could cleave when the site is positioned closer to the PTC. We examined a variety of nascent chains of different lengths. After translation in wheat germ lysate system, samples were divided. Factor Xa was directly added to one portion where cytosol had not been removed. In the other half, cytosol was depleted from the nascent chain-ribosome complexes by high salt extraction and sedimentation before factor Xa addition. When factor Xa was added to nascent chains having cleavage sites far from the PTC (>33 amino acids), nascent chains could be cleaved in the presence of cytosol (Table I). As expected, short nascent chains with cleavage sites 27, 24, 18, 15, or 12 amino acids from the PTC were not cleaved when factor Xa was directly added to the translation mixtures. Surprisingly, after cytosol depletion by high salt extraction, factor Xa could cleave all nascent chains tested (see Table I).

Fig. 2 B shows the cleavage of the shortest nascent chain tested. 32aaSM-1 mRNA encodes a 32-amino acid nascent chain with factor Xa cleavage site 12 amino acids from the PTC (see Fig. 1). When factor Xa was added without any further manipulation, the nascent chain was not cleaved (Table I). After high salt extraction and sedimentation through a high salt sucrose cushion to remove nonribosomal proteins, factor Xa could cleave (Fig. 2 B, lane 2). This result indicates that a large aqueous probe (43 kD) can gain access to regions of nascent chains as near as 12 amino acids from the PTC after removal of cytosolic factors.

It was important to establish that after factor Xa cleavage of the 32-amino acid nascent chain, the carboxy-terminal 12 amino acid digested product remained associated as a peptidyl tRNA on the ribosome. Cetyl-trimethyl ammonium bromide (CTABr), a cationic detergent that precipitates RNA but not protein, precipitated the 12-amino acid-peptidyl-tRNA (Fig. 2 B, lane 3). This indicates that we were examining cleavage of ribosome-associated nascent chains. Fig. 2 C demonstrates that salt extraction does not irreversibly impair the ability of the ribosomes to effect translational elongation. Translation of 116aaSM-1 in the wheat germ lysate system was stopped after one minute. After high salt extraction, the nascent chain-ribosome complexes were isolated and resuspended in translation blank buffer (TBB). Excess nonradioactive methio-

nascent chains to their full-length (lane 2). Proteins were directly applied to 15% SDS-PAGE without TCA precipitation and visualized by fluorography.

Table 1. Accessibility of Nascent Chains to Factor Xa Cleavage

Distance*	Xa Cleavage	
	Non-high-salt stripped [‡]	High-salt stripped [§]
<i>aa</i>		
>100	+	+
58	+	+
47	+	+
44	+	+
42	+	+
41	+	+
40	+	+
39	+	+
38	+	+
37	+	+
36	+	+
35	+	+
33	+	+
27	-	+
25	-	+
24	-	+
23	-	+
19	-	+
18	-	+
15	-	+
12	-	+

*Number of amino acids between the PTC to the factor Xa cleavage site.
[‡]Factor Xa was added directly to the nascent chain-ribosome complexes.
[§]Factor Xa was added after nascent chain-ribosome complexes were treated with high salt and isolated through a high salt sucrose cushion.

nine together with a complete wheat germ translation mixture were added. One-half of the sample was kept on ice (Fig. 2 C, lane 1) and the other half was incubated for 15 min to elongate the short 20–30-amino acid long nascent chains to full-length 116-amino acid polypeptides (Fig. 2 C, lane 2). Also the presence of factor Xa did not impair translational elongation (not shown).

NAC Protects Short Nascent Chains from Proteolysis

We next sought to determine whether readdition of purified NAC is capable of restoring protease protection of the nascent chain. For the experiment shown in Fig. 3, 32aaSM-1 and 44aaSM-1 mRNAs, where the factor Xa cleavage sites are 12 and 24 amino acids from the PTC, respectively, were translated in the wheat germ system. Nascent chain-ribosome complexes were salt extracted and isolated by centrifugation. After resuspension in TBB, samples were divided into aliquots. When added directly, factor Xa could cleave both nascent chains (Fig. 3, compare lanes 1 with 2). In contrast when NAC-containing cytosol from wheat germ (Fig. 3, lanes 3 and 4) or bovine brain (Fig. 3, lanes 7 and 8) was returned to the stripped nascent chains before factor Xa addition, a dose dependent protection from digestion was observed. This demonstrates that the loss of protection observed upon cytosol depletion is reversible. Since NAC binds nascent chain-ribosome complexes reversibly and it is in contact with short nascent chains, we asked whether purified NAC, near its physiological concentration, could afford protection from factor Xa digestion. Indeed, when purified NAC was added to stripped chains before incubation with factor Xa, protection was observed (Fig. 3, lanes 5 and 6). Strikingly, we found that a cytosol depleted of NAC to greater

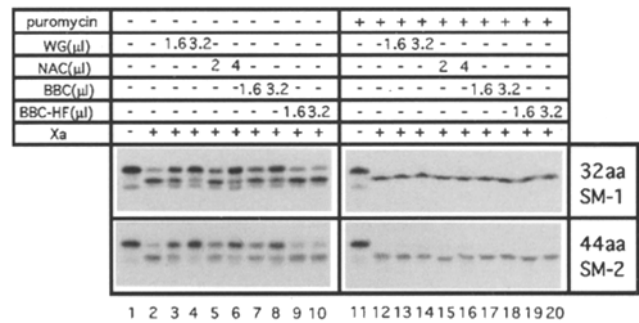


Figure 3. NAC protects short nascent chains from proteolysis. After in vitro translation of 32aaSM-1 or 44aaSM-2 in the wheat germ lysate system supplemented with [³⁵S]methionine, samples were extracted with high salt and nascent chain-ribosome complexes were isolated by centrifugation through a high salt-containing sucrose cushion. After resuspension in TBB, samples were divided. Factor Xa was directly added to the nascent chain-ribosome complexes (lane 2) or omitted (lanes 1 and 11). Wheat germ lysate (*WG*, lanes 3, 4, 13, and 14), purified NAC (lanes 5, 6, 15, and 16), bovine brain cytosol (*BBC*, lanes 7, 8, 17, and 18), or NAC-depleted bovine brain cytosol (*BBC-HF*, lanes 9, 10, 19, and 20) was added before addition of factor Xa. After mixing components, samples were kept at room temperature for 10 min and then on ice for 20 min before factor Xa digestion on ice for 20 min. A parallel set of reactions was treated with puromycin (1 mM, 37°C, 10 min), and RNase A (1 mg/ml, 37°C, 10 min) before factor Xa addition (lane 11–20). Sample volumes were 10 μ l. After reaction, samples were directly denatured in sample buffer and separated on a 15.5% acrylamide tricine gel.

than 95% but to less than 10% of total protein, was not able to protect nascent chains from digestion (Fig. 3, lanes 9 and 10). This indicates that NAC is likely to be the major protecting factor present in the cytosol. Note that when a parallel set of samples was treated with puromycin and RNase A to release the nascent chains before factor Xa addition, no protection was observed, indicating that our experimental manipulations had no nonspecific inhibitory effects on the ability of the protease to function.

These data demonstrate that nascent chains do not traverse a tunnel of 35–40 residues in length composed of core ribosomal proteins, as regions of nascent chains can be accessible to a large proteinase probe very soon after synthesis. NAC protects about the 30 COOH-terminal amino acids of a nascent chain. Next using the same constructs, we examined the molecular environment from the perspective of the nascent chains using the photocross-linking technique.

NAC Cross-links to Very Short Nascent Chains

Identified using the photocross-linking technique, NAC was recently described as a cytosolic protein that interacts exclusively with ribosome-associated (as opposed to terminated and released) nascent chains (Wiedmann et al., 1994) that is present in a variety of cytosols in greater abundance than ribosomes (H. Sakai, S. Wang, and M. Wiedmann, manuscript in preparation). NAC is a heterodimeric protein complex containing two subunits, α and β NAC. Because NAC is in contact with regions of nascent chains as near as 35 amino acids from the carboxy

terminus (or PTC), we reasoned that NAC is likely to be one of the first cytosolic proteins that a newly synthesized polypeptide encounters (Wiedmann et al., 1994). Using the constructs described above with known cross-linking sites, we sought to determine both how near to and far away from the PTC NAC could be cross-linked to nascent chains.

Fig. 4 A shows a typical result from a NAC cross-linking experiment. An mRNA encoding a 56-amino acid version of SM-3 (see Fig. 1) was produced and translated in a wheat germ system in the presence of TDBA-lys-tRNA and [³⁵S]methionine. In this nascent chain, lysines are located at amino acid positions 5, 8, and 9 from the NH₂ terminus; therefore the lysine nearest to the carboxy terminus is 47 amino acids away from the PTC. We therefore expected to obtain cross-links to cytosolic rather than ribosomal proteins. After translation, samples were irradiated to induce cross-linking. As expected (Wiedmann et al., 1994), two major cross-linking products were observed to be α and βNAC (lane 2). Salt extraction of the samples and isolation of the salt stripped nascent chain-ribosome complexes by centrifugation through high salt-containing sucrose cushions before cross-linking results in a decrease in the intensity of the NAC cross-links (Fig. 4 A, compare lane 2 with 3). This demonstrates once again that NAC can be extracted by high salt from nascent chain-ribosome complexes. When purified mammalian NAC was added to the high-salt stripped nascent chain-ribosome complexes before irradiation, the intensity of the NAC cross-links

dramatically increased (Fig. 4 A, lane 5). Because the mammalian αNAC is 2–3 kD larger than the wheat germ αNAC, a molecular mass shift in NAC cross-links is evident (Fig. 4 A, compare lane 2 with 5; Wiedmann et al., 1994).

With the ability to synthesize a variety of nascent chains of different lengths in either wheat germ lysate or reticulocyte lysate translation system, and since these nascent chains only have lysines in their NH₂ termini, we could determine which regions of a nascent chain are in contact with NAC. NAC could be cross-linked to nascent chains over a wide range of distances from the PTC—17, 27, 30, 33, 34, 35, 36, 38, 43, 44, 46, 47, 49, 53, 55, 58, and 100 amino acids from the PTC (not shown). Therefore NAC can interact with regions of nascent chains as far away as ~100 amino acids from the PTC. We were surprised to discover that NAC, which is not a core ribosomal protein, could also cross-link as close as 17 amino acids from the PTC, for it is generally believed that nascent chains do not emerge from their putative ribosomal tunnel until after 35–40 residues are synthesized (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Bernabeu and Lake, 1982; Bernabeu et al., 1983).

For the experiment shown in Fig. 4 B, 32aaSM-1 and 22aaSM-1 mRNAs, where the lysines furthest from the PTC are 27 and 17 amino acids, respectively, were translated in vitro. Stripped nascent chain-ribosome complexes were then isolated and resuspended in TBB. Upon irradiation, both αNAC and βNAC cross-links were detectable

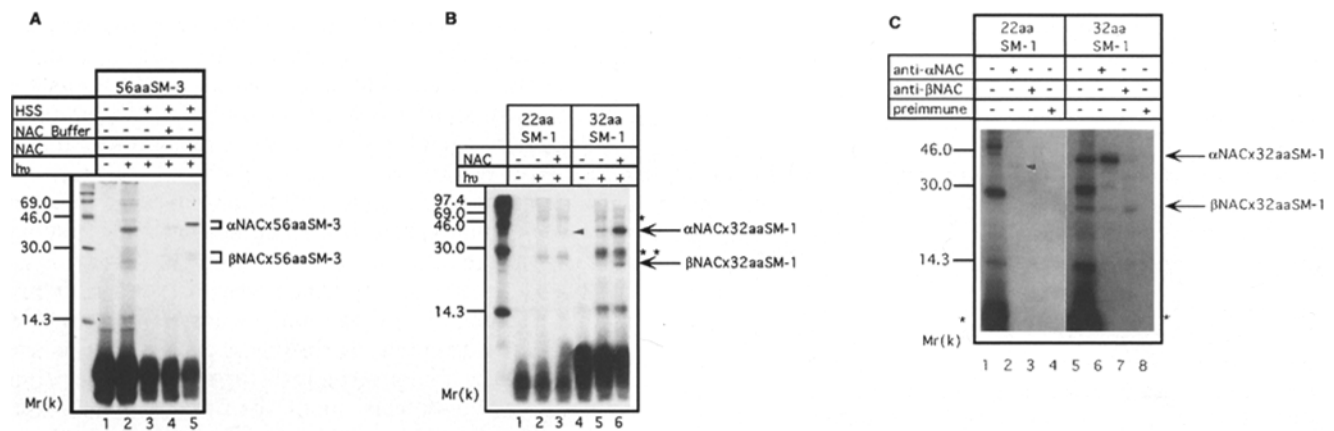
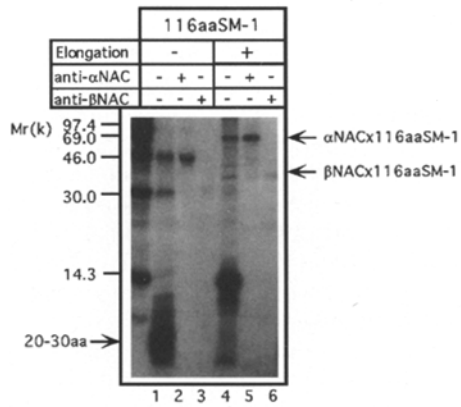


Figure 4. NAC cross-links to very short nascent chains. (A) 56aaSM-3 transcripts were translated in the wheat germ lysate system supplemented with [³⁵S]methionine and TDBA-lys-tRNA. The sample was divided so that one-fifth was kept as a nonirradiated control (lane 1) and one-fifth was irradiated (lane 2). Three-fifths of the sample not yet subjected to cross-linking was extracted with high salt, and nascent chain-ribosome complexes were isolated through a high salt sucrose cushion. After resuspension in TBB, samples were irradiated directly (lane 3), after adding NAC buffer (lane 4), or after adding bovine brain NAC (200 nM, lane 5). Proteins were separated by SDS-PAGE on a 15% SDS-PAGE and visualized by fluorography. (B) Transcripts encoding 22aaSM-1 or 32aaSM-1 were translated in the reticulocyte lysate system supplemented with [³⁵S]methionine and TDBA-lys-tRNA. Samples were treated with high salt and nascent chain-ribosome complexes were isolated by centrifugation through a high salt sucrose cushion. After resuspension in TBB, a portion of the sample was not irradiated as a control (lanes 1 and 4) or irradiated to induce cross-linking (lanes 2 and 5). For the samples shown in lanes 3 and 6, NAC was added before irradiation at a concentration of 200 nM. The arrowhead denotes the cross-link of αNAC to 22aaSM-1. * and ** indicate cross-links of the nascent chains to putative ribosomal proteins. Proteins were separated by 15% SDS-PAGE and visualized by fluorography. (C) 22aaSM-1 and 32aaSM-1 were translated in the reticulocyte lysate system supplemented with [³⁵S]methionine and TDBA-lys-tRNA. Nascent chain-ribosome complexes were isolated by centrifugation through a low salt sucrose cushion, resuspended in TBB, and irradiated (lanes 1 and 5). Irradiated samples were subjected to immunoprecipitation with either NAC-specific antibodies (lanes 2, 3, 6, and 7) or preimmune serum (lane 4 and 8). The arrowhead indicates the cross-links of αNAC to 22aaSM-1. The asterisk shows the position of the nascent chains. Proteins were separated by 15% SDS-PAGE and visualized by fluorography.

A



B

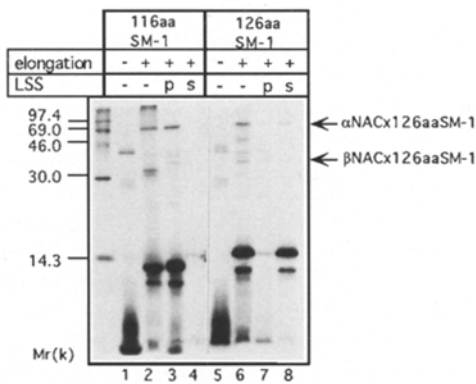


Figure 5. Nascent chains covalently cross-linked to NAC can be elongated by and released from the ribosome. (A) To test whether, after being cross-linked to NAC, short nascent chains can be further elongated, the following experiment was performed. Transcripts encoding 116aaSM-1 were translated for one minute in reticulocyte lysate system supplemented with [35 S]methionine and TDBA-lys-tRNA. The translation mixture was rapidly cooled on ice, and the ribosome complexes containing very short nascent chains were isolated by centrifugation through a low salt sucrose cushion. The nascent chain-ribosome complexes were resuspended in TBB and irradiated. Then reticulocyte lysate translation mixture containing excess nonradioactive methionine and normal lysine was added to the nascent chain-ribosome complexes. Samples were divided, and one-half was kept on ice as a control (lane 1). The other half was incubated at 26°C for 15 min (lane 4). Samples shown in lanes 1 and 4 were precipitated by ammonium sulfate. Samples shown in lanes 2, 3, 5, and 6 were immunoprecipitated by NAC-specific antibodies after ammonium sulfate precipitation to identify the products. Proteins were separated by 15% SDS-PAGE and visualized by fluorography. (B) Covalent attachment of NAC during translation does not interfere with termination and release of the polypeptide. As in the experiment in Fig. 6 A, 116aaSM-1 and 126aaSM-1 were translated for 1 min at 26°C in the reticulocyte lysate system and elongated after cross-linking by addition of complete reticulocyte lysate translation mixture containing excess nonradioactive methionine and normal lysine (lanes 1, 2, 5, and 6) at 26°C for 15 min. Elongated 116aaSM-1 and 126aaSM-1 translation mixtures were centrifuged through a low salt sucrose cushion. Pellet (P, lanes 3 and 7) and supernatant fractions (S, lanes 4 and 8) were recovered.

when 32aaSM-1 was used (Fig. 4 B, lane 5). When purified NAC was returned to the samples at approximately its original concentration, the intensities of NAC cross-linked bands increased (Fig. 4 B, lane 6). For 22aaSM-1, the α NAC cross-link was readily detected (arrowhead) while the β NAC cross-link could only be seen after very long exposures of the fluorograph. The identity of the α and β NAC cross-links were confirmed by immunoprecipitation using anti-NAC antibodies (Fig. 4 C). Also notice that there are other major cross-linking bands besides NAC cross-links (labeled * and **) that, unlike NAC, were neither extracted by high salt (Fig. 4 B, lanes 2 and 5) nor released with the nascent chains upon puromycin addition (not shown). Therefore we believe that these are cross-links to core ribosomal proteins.

Nascent Chains Covalently Cross-linked to NAC Can Be Elongated by and Released from the Ribosome

For the experiment depicted in Fig. 5 A, we wished to know whether the nascent chain with NAC cross-linked can still be elongated. Our experimental strategy was to start a translation reaction, temporarily halt translational elongation, covalently cross-link NAC to the nascent chain, and finally continue elongation and determine whether the chain, when cross-linked to NAC, is still capable of elongation.

116aaSM-1 mRNA was translated in vitro in the reticulocyte lysate system supplemented with [35 S]methionine and TDBA-lys-tRNA for 1 min—just long enough for initiation and synthesis of 20–30 amino acids. Translational elongation was then halted and nascent chain-ribosome complexes were immediately centrifuged through a low salt-containing sucrose cushion. After resuspension in TBB, samples were irradiated to covalently cross-link NAC to very short nascent chains. Fig. 5 A (lanes 1–3) shows these NAC cross-links. A fresh reticulocyte translation mixture containing excess nonradioactive methionine and normal lysine was added. One half was kept on ice as a control (Fig. 5 A, lanes 1–3) whereas the other half was incubated at 26°C for 15 min to allow elongation of the stalled nascent chains to proceed (Fig. 5 A, lanes 4–6). As is seen, when elongation occurs, the halted nascent chains grow to their full length. Surprisingly the NAC cross-links also move to high positions on SDS-PAGE (Fig. 5 A, compare lane 1 with 4). This “growth” of the cross-links indicates that even after covalent attachment of NAC, the elongation machinery is intact and functional. These data are consistent with NAC being intimately associated with nascent chains and not simply forming a rigid roof under which nascent chains pass. The identity of the cross-linked species was confirmed by immunoprecipitation using NAC-specific antibodies (Fig. 5 A, lanes 2, 3, 5, and 6). Covalent attachment of NAC to the nascent chain does not interfere with polypeptide elongation.

We next asked whether the NAC-nascent chain cross-link could be released from the ribosome upon reading a termination codon. Two similar mRNAs, one lacking a stop codon (116aaSM-1) and the other having a stop codon

Proteins were separated by 15% SDS-PAGE and visualized by fluorography.

(126aaSM-1) were translated in the reticulocyte translation system supplemented with [³⁵S]methionine and TDBA-lys-tRNA for one minute (Fig. 5 B). As in Fig. 5 A, translation was stopped after one minute and nascent chain-ribosome complexes were recovered and resuspended in TBB. Samples were irradiated to induce cross-linking and divided. One portion remained nonelongated as a control (Fig. 5 B, lanes 1 and 5) while in the other samples, elongation was restored (Fig. 5 B, lanes 2 and 6). As expected, the cross-links to NAC "grow." When elongated samples were further examined by fractionation of ribosomes from the cytosol by centrifugation through low salt sucrose cushions, the nascent chain-NAC cross-links were recovered with the ribosomes only when the mRNA lacked a termination codon (Fig. 5 B, lane 3). In contrast, when the mRNA encoded a stop codon, the chains were terminated and a substantial fraction of the NAC-nascent chain cross-links was released into the supernatant and no longer fractionated with the ribosomes (Fig. 5 B, lane 8). These findings suggest that whatever affinity NAC has for the ribosome, this does not prevent release of the cross-link upon termination.

Discussion

Many investigators have addressed the question of how nascent chains emerge from the ribosome. Protease protection experiments (Malkin and Rich, 1967; Blobel and Sabatini, 1970), where randomly assembled polysomes in the presence of complete cytosol were subjected to proteolysis with nonspecific proteases, revealed that fragments of nascent chains ~35–40 amino acids long were protected from proteolysis. It was concluded that these fragments corresponded to COOH-terminal peptidyl-tRNAs protected by a putative ribosomal tunnel. Bernabeu and Lake tried to identify the exit site for nascent chains on ribosomes using immunoelectron microscopy (Bernabeu and Lake, 1982; Bernabeu et al., 1983). Their studies showed that β -galactosidase nascent chains on *E. coli* ribosomes and ribulose-1,5-bisphosphate-carboxylase nascent chains on plant ribosomes first became accessible to antibody labeling in a region located at the base of the large ribosome subunit opposite the central protuberance, the presumed location for PTC. They further estimated the distance from the PTC to the nascent chain exit at 150 ± 30 Å, and assumed that this was the length of the tunnel. To meet the 150 ± 30 Å long tunnel requirement, they proposed that a nascent chain must be fully extended in the ribosome.

A low electron density region of 150–200 Å in length, and again a presumed tunnel, was reported within the large subunit of reptilian ribosomes by reconstitution of the two-dimensional images of electron microscopy of ribosomes (Milligan and Unwin, 1986). Later, Yonath et al. (1987) used a similar technique and also identified an elongated zone of low electron density inside the 50S ribosomal subunit. The zone, also proposed to be a nascent chain tunnel, was ~100–120 Å in length and 25 Å in diameter. From all of this work, the existence of a long intraribosomal tunnel formed by core ribosomal proteins for a nascent chain has been widely accepted. In no case, how-

ever, has a nascent chain been localized to these low-density regions (Yonath and Wittmann, 1989).

Nevertheless, there are data that suggest that no such tunnel exists. Palmiter et al. (1978) reported that in a growing ovalbumin peptide the NH₂-terminal initiator methionine was removed by an aminopeptidase after just 15–20 amino acids had been synthesized. Furthermore, NH₂-terminal acetylation can proceed on a nascent chain of ~25 amino acids in length (Strous et al., 1974). Cotranslational N-glycosylation was also found to occur on positions of nascent chains too short to span a long tunnel in the ribosome (Glabe et al., 1980). Ryabova et al. (1988) also used immunoelectron microscopy to map the exit site on translating ribosomes. Using an antibody directed against a derivatized initiator methionine, they found that the nascent chains were almost immediately accessible to antibodies, suggesting that there was no tunnel at all. They proposed that (a) there was no intraribosomal tunnel for nascent chains; (b) that nascent chains were almost immediately accessible to the cytosol; and (c) the path for a nascent chain might be the groove on the surface of the large ribosomal subunit.

More recently, Mothes et al. (1994), using the same cross-linking technique utilized in this study, found that a nascent secretory polypeptide chain can be cross-linked to the integral ER membrane protein Sec 61p when the only cross-linker was positioned as close as 26 amino acids from the COOH terminus.

We believe that some of the apparent contradictions in these various studies may result from the fact that the existence of factors such as NAC was not known. In this work we have demonstrated that NAC is likely to be among the first cytosolic factors that a newly synthesized nascent chain encounters, for it can be cross-linked as near as 17 amino acids from the PTC. Because its binding to nascent chain-ribosome complexes is reversible, NAC is best considered as a cytosolic factor, as core ribosomal proteins are those whose association with the ribosomes are stable in the presence of high salt. Then, NAC, like translation elongation factors, can be regarded as part of the translation machinery.

Although low, the cross-linking efficiency NAC to 22aaSM-1 nascent chain is not an indicator of the percentage of nascent chains that are in contact with NAC (Görllich et al., 1991). Nonetheless, the technique is useful in obtaining information on qualitative aspects of environment of the nascent chains.

We believe that nascent chains are indeed normally segregated from the cytosol until ~30 amino acids in length, as suggested by others. However, we believe that this protection is not as a result of sequestration within a tunnel comprised of core ribosomal proteins, but instead occurs because NAC is associated to these nascent chains. Even though factor Xa, in the presence of NAC, cannot gain access to regions of nascent chains nearer than ~30 amino acids from the PTC, other factors like modifying enzymes that can cotranslationally interact with emerging polypeptides may, by virtue of an affinity for nascent chain-ribosome complexes, be able to interact with regions of nascent polypeptides nearer than 30 amino acids from the PTC. We have demonstrated that in the presence of cytosol or purified NAC, chains are protected from controlled

proteolysis up to ~30 amino acids in length. When NAC is removed, the chains become susceptible to proteolysis at ~12 amino acids in length. Due to the technical difficulty of resolving peptides shorter than 12 amino acids on SDS-PAGE, the factor Xa accessibility of nascent chains with cleavage sites less than 12 amino acids from the PTC was not addressed. Current models suggest that the rigid tunnel starts at PTC and ends at the nascent chain exit site, and it spans the entire large subunit of a ribosome (~150 Å in length). Thus even if there is a rigid tunnel comprised of core ribosomal proteins, the length of the tunnel should be less than 50 Å (as judged by the maximal length of a fully extended 12-amino acid peptide segment). Readdition of purified NAC results in protection of chains whose length is similar to those protected in previous proteolysis experiments (Malkin and Rich, 1967; Blobel and Sabatini, 1970). Thus NAC, by covering a nascent chain, gives the appearance of the existence of the putative ribosomal tunnel. We imagine that in previous proteolysis experiments, NAC was present on the nascent chains studied. Because we can detect NAC being released with nascent chains *in vitro* and because NAC is in stoichiometric excess compared to ribosomes (H. Sakai, S. Wang, and M. Wiedmann, manuscript in preparation), we believe that a NAC cycle of binding and release occurs. Clearly, more work will be needed to unambiguously demonstrate the existence of such a phenomenon.

We propose that ribosomal exit is coincident with displacement of NAC from a particular region of the nascent chain. Fidelity of nascent chains' folding, modification, and targeting might require exposing nascent chains domain by domain rather than amino acid by amino acid. This could be accomplished if cytosolic factors bind to discrete regions of nascent chains almost immediately after synthesis and remain associated until being subsequently released in the cytosol. When nascent chains are sufficiently far from the PTC, NAC's affinity for the chains decreases and it either falls off or is displaced by factors involved in targeting or folding. Although NAC can be cross-linked to regions as near as 17 and as far away as 100 amino acids from the PTC, NAC could only prevent proteolysis by factor Xa when the recognition site was closer than ~30 amino acids from the PTC. This observation suggests that NAC binds to nascent chains more tightly when near the PTC, and in this helps to prevent premature and inappropriate interactions with cytosolic nascent chain interacting factors.

Because NAC can be shown to be in direct contact with nascent chains by the photocross-linking approach, we do not favor the idea that NAC binding alters the conformation of the ribosome in a way that now allows ribosomal proteins to shield the nascent chains. The environment of nascent chains on ribosomes has been suggested to be hydrophilic (Crowley et al., 1993). We propose that NAC association with ribosome-associated nascent chains, together with the ribosome, forms this aqueous environment. Nascent chains may either fill a groove on the surface of a ribosome or occupy a large cavity in the ribosome. In either case NAC would separate the nascent chain from the cytosol. We imagine that the reversibility of NAC association with ribosomes might allow for a flexible, dynamic exit site for newly synthesized polypeptides.

We thank B. Lauring for discussion and assistance in writing the manuscript. We also thank Drs. F.-U. Hartl, K. van Leyen, J. Rothman, G. Tanigawa, B. Wiedmann, and members of the Wiedmann and Rothman laboratories for critically commenting on the manuscript.

Received for publication 17 March 1995 and in revised form 4 May 1995.

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