Interaction of the Eukaryotic Elongation Factor 1A with Newly Synthesized Polypeptides*

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The abbreviations used are: NC, nascent chain; NAC, nascent chain-associated complex; RAF, ribosome-associated factor; SRP, signal recognition particle; RNC, ribosome nascent-chain complex; flLuc, firefly luciferase; α, α-amino acids; EF-Tu, elongation factor Tu; eEF1A, eukaryotic elongation factor 1A; formerly known as EF1α; GST, glutathione S-transferase; GST-eEF1A, GST-eEF1A fusion protein; DTT, dithiothreitol; pPL, pre-prolactin; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; RRL, rabbit reticulocyte lysate.

*eEF1A, the eukaryotic homologue of bacterial elongation factor Tu, is a well characterized translation elongation factor responsible for delivering aminoacyl-tRNAs to the A-site at the ribosome. Here we show for the first time that eEF1A also associates with the nascent chain distal to the peptidyltransferase center. This is demonstrated for a variety of nascent chains of different lengths and sequences. Interestingly, unlike other ribosome-associated factors, eEF1A also interacts with polypeptides after their release from the ribosome. We demonstrate that eEF1A does not bind to correctly folded full-length proteins but interacts specifically with proteins that are unable to fold correctly in a cytosolic environment. This association was demonstrated both by photo-cross-linking and by a functional refolding assay.

Newly synthesized proteins first encounter the crowded cytosolic environment where total protein concentrations can be as high as 500 mg/ml (1, 2) when they are still nascent chains (NCs) being translated on the ribosome. These NCs then have to find their way to their proper destination (3) and adopt the one correct conformation of the many that are possible (4) while avoiding inappropriate interactions with other cytosolic proteins. To ensure fidelity in this highly complex process, eukaryotic cells contain sophisticated targeting and translocation machinery for the transport of proteins to different intracellular destinations and a system of chaperones and chaperonins to help the protein fold correctly. Both protein transport and folding have been major topics of research for the last decade, and we have a fairly detailed understanding of both processes (4–9). Nevertheless, recent findings demonstrate that up to 50% of all newly synthesized polypeptides are immediately degraded and emphasize how error prone and complicated the synthesis and maturation of proteins actually must be (10, 11).

Many of the proteins that oversee the maturation of newly synthesized polypeptides associate with the ribosome during growth of the NC. To the best of our knowledge, nascent polypeptide-associated complex (NAC) is the first non-ribosomal protein that the growing NC encounters. Its major function is to shield the NC from premature encounters as it emerges from the ribosome. NAC is also involved in regulating ribosome binding to the endoplasmic reticulum membrane (12–14) and in mitochondrial protein import (15, 16).

As the NC lengthens, NAC binding can be competed by other ribosome-associated factors (RAFs) such as signal recognition particle (SRP). NAC, which is highly abundant, has a low affinity for the ribosome nascent-chain complexes (RNCs), regardless of which NC is being translated. Unlike NAC, SRP is of low abundance (20 nm) but has high affinity ($K_d \sim 10^{-13}$ M) specifically for the hydrophobic residues present in a signal peptide (17). SRP binding is essential for co-translational translocation into the lumen of the ER and entrance of proteins into the secretary pathway (6).

Many proteins that remain in the cytosol must bind to chaperones such as HSP70/40 to avoid inappropriate aggregation and acquire the correct conformation. Chaperones may be able to bind to NCs as they emerge from the ribosome but clearly can also associate with proteins post-translationally and can also protect proteins that are denatured under conditions of stress. Members of the HSP70 family of chaperones are also involved in post-translational transport of proteins into the ER and mitochondria (7, 8, 18).

Several of the known RAFs, including SRP and NAC, were initially characterized as associating with NCs by demonstrating that they could be photo-cross-linked to NCs (19, 20). Here, we identify and characterize a 50-kDa RAF as eEF1A. eEF1A is implicated in several cellular processes. It is well known to play a principal role in translation by catalyzing GTP-dependent binding of aminoacyl-tRNA to the A-site at the ribosome. eEF1A has also been demonstrated to bind actin (21) and to sever microtubules, a prerequisite for cytoskeletal rearrangements that occur during the cell cycle (22). Here we show that eEF1A binds to RNCs and to unfolded polypeptides that are no longer associated with the ribosome but not to correctly folded proteins. Based on these data as well as previous reports that eEF1A stimulates ubiquitin-dependent degradation of N-acetylated proteins (23), we suggest that eEF1A may also play a role in quality surveillance of newly synthesized proteins.

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### MATERIALS AND METHODS

**Plasmids**—The truncated polypeptide and full-length proteins used in these studies are summarized in Table I, which notes the positions of all methionines and lysines and the restriction enzymes used to produce the truncated templates. Firefly luciferase (ffLuc) polypeptides are encoded by the plasmid pT3-luciferase, a gift of Dr. M. Strauss. Wild-type bovine pre-prolactin (pPL) is encoded by pSPBP4, whereas pPL-M is encoded by pEGM4-PPL/SSKO, and they are gifts of Drs. P. Walter and B. Dobberstein, respectively. In pPL-M, the signal peptide has been deleted, and then treated with RNase A and analyzed on 6% NuPAGE Bis-Tris gel in MES buffer and visualized by autoradiography (XAR-5, Eastman Kodak Co.) except as noted.

**Bis-Tris gel in MES buffer and visualized by autoradiography (XAR-5, Eastman Kodak Co.)**

**Release of Polypeptide Chains from the Ribosome**—RNCs were incubated on ice for 30 min with 2 mM puromycin and then at 26°C for 10 min with 0.1 mg/ml RNase A to release the newly synthesized polypeptides. After treatment, the reaction mixture was centrifuged (100,000 rpm at 4°C for 20 min, TLA 120.1 rotor) through a low salt-sucrose cushion (0.5 M sucrose in translation blank buffer) to separate ribosomes (pellet) from released polypeptides and RAPs (supernatant).

**Purification of eEF1A**—eEF1A was isolated from rabbit reticulocyte lysate prepared as described previously (28). After sedimentation of the ribosomes by centrifugation for 60 min at 100,000 rpm in a Beckman rotor TLA 100.4, proteins were precipitated with 66% ammonium sulfate, dialyzed against buffer A overnight, and applied to a Q-Sepharose column (Amersham Biosciences). The flow-through was applied to an S-Sepharose column (Amersham Biosciences) and eluted with a linear gradient of 10–1000 mM potassium acetate in buffer A. A single protein was obtained at 390–450 mM potassium acetate concentration. The isolated protein was digested against ap2ase and analyzed on 6% NuPAGE Bis-Tris gel in MES buffer and visualized by autoradiography (XAR-5, Eastman Kodak Co.) except as noted.

**Isolation of the High Salt-stripped Nascent Chains and Cross-linking Assay**—To strip RAPs from ribosomes, translation reactions were diluted into 10 volumes of ice—cold high salt buffer (50 mM Hepes-KOH, pH 7.5, 700 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, protease inhibitor mixture as described by Erickson and Blobel (28), and 0.4 units/μl plasmid RNase inhibitor), and the high salt stripping was repeated as described above to completely remove associated proteins. The isolated high salt-striped RNCs were incubated at 26°C for 10 min with buffer A (50 mM Hepes-KOH, pH 7.5, 50 mM potassium acetate, 5 mM magnesium acetate, and 1 mM DTT), rabbit reticulocyte lysate (precipitated with 66% ammonium sulfate and dialyzed against buffer A), or purified protein in buffer A supplemented with RNase inhibitor and protease inhibitors.

**Protein nomenclature and positions of relevant amino acids**

| Protein          | Positions of Methionines | Positions of Lysines | Restriction enzyme |
|------------------|--------------------------|----------------------|-------------------|
| 85 aa β-actin    | 1, 16, 44, 47, 82        | 18, 50, 61, 68, 84   | Bgl II            |
| 133 aa β-actin   | 1, 16, 44, 47, 82, 119, 123| 18, 50, 61, 68, 84, 113, 118 | SnaBI |
| 221 aa β-actin   | 1, 16, 44, 47, 82, 119, 123, 132, 153, 190| 18, 50, 61, 68, 84, 113, 118, 191, 213, 215 | XbaI |
| 77 aa ffLuc      | 1, 30, 59, 67           | 5, 8, 9, 28, 31, 68  | HinII             |
| 197 aa ffLuc     | 1, 30, 59, 67, 90, 118, 152, 164, 196 | 5, 8, 9, 28, 31, 68, 130, 131, 135, 141, 142, 148, 155, 190 | EcoRI |
| 550 aa ffLuc     | 1, 30, 59, 67, 90, 118, 152, 164, 196, 249, 265, 396, 398, 493 | 5, 8, 9, 28, 31, 68, 130, 131, 135, 141, 142, 148, 155, 190, 206, 281, 297, 303, 321, 329, 358, 364, 372, 380, 414, 439, 443, 445, 491, 496, 510, 511, 529, 534, 541, 543, 544, 547, 549 | * |
| 229 aa pPL-M     | 1, 54, 66, 83, 111, 135, 160, 162 | 4, 9, 72, 78, 99, 136, 154, 172, 189, 211, 217, 217 | * |
| 86 aa pPL-MN     | 1, 54, 66, 83           | 4, 9                 | PvuII             |
| 169 aa pPL-MN    | 1, 54, 66, 83, 111, 135, 160, 162 | 4, 9                 | EcoRI             |
| 229 aa pPL-wt     | 1, 54, 66, 83, 111, 135, 160, 162 | 4, 9, 72, 78, 99, 136, 154, 172, 189, 211, 217 | * |

**Recombinant Protein Expression and Purification**—A plasmid encoding a GST–eEF1A fusion protein is a generous gift of J. Condeelis (29). *Escherichia coli* DH5α cells were transformed with this plasmid, and a 2-liter culture in 2× YT was incubated at room temperature until it reached an optical density of 0.8 (600 nm) followed by the addition of 1% isopropyl-β-D-thiogalactopyranoside to 0.2 mM. After 7–8 h, the cells were collected by centrifugation, resuspended in 100 ml of buffer (50 mM Tris–HCl, pH 8.0, 300 mM KCl, 15% glycerol, 1% Triton X-100, 1 mM DTT, and protease inhibitor), and broken by one pass through an Avestin cell disrupter (Ottawa, Canada) at greater than 10,000 p.s.i.

After centrifugation of the lysate at 12,000 rpm for 10 min in a SA600 rotor (Sorvall), the resulting supernatant was spun at 35,000 rpm in a Ti45 rotor (Beckman) for 60 min. The supernatant was then incubated overnight at 4°C with 2 ml of glutathione-Sepharose 4B beads (Amersham Biosciences). After centrifugation and wash, the beads were resuspended in 1 ml of glutathione elution buffer (Amersham Biosciences) and incubated at room temperature for 10 min to elute the GST–eEF1A fusion protein from the beads. Finally, the beads were sedimented, and the GST–eEF1A fusion protein was recovered from the supernatant. To cleave the GST from eEF1A, the GST–eEF1A protein was treated with thrombin (4 units/ml) and incubated at room temperature for 16 h. Thrombin was inactivated by the addition of phenylmethylsulfonyl fluoride (1 mM) and AEBSF (0.5 mM) for 30 min followed by dialysis against buffer A containing 15% glycerol. Recombinant eEF1A protein was recovered from the supernatant after incubation of the mixture with glutathione-Sepharose 4B beads.
Reduced intensity of both cross-linking products to the level of the non-irradiated control (Fig. 1, compare lanes 1 and 4). The addition of rabbit reticulocyte lysate to the high salt-stripped RNCs before irradiation restored the cross-link to the 50- and 120-kDa proteins (Fig. 1, compare lanes 2 and 5). Collectively, these results indicate that the 50- and 120-kDa proteins are salt-extractable proteins and can rebind to the 169 aa pPL-MN NC after extraction. The rebinding of the 50-kDa protein provided an assay that we have used for its purification.

**Purification of 50-kDa NC Associated Protein**—The 50-kDa protein that cross-links to 169 aa pPL-MN NC was purified from rabbit reticulocyte lysate as follows. The supernatant from a high speed centrifugation to sediment the ribosomes was precipitated with 66% ammonium sulfate, dialyzed, and applied to a Q-Sepharose column. The flow-through, which contained the 50-kDa cross-linking partner (Fig. 2A), was applied to an S-Sepharose column and eluted with a gradient of potassium acetate (10–1000 mM, the fraction shown is 390–450 mM). Fig. 2A, lanes 1–4, shows the silver stain of the protein pattern after SDS-PAGE for the different purification steps. In addition, samples were assayed for cross-linking to 169 aa pPL-MN NCs as shown in Fig. 2A, lanes 5–10. High salt-stripped RNCs irradiated in buffer alone (Fig. 2A, lane 6) show that the removal of the 50-kDa cross-linking partner was efficient. Fig. 2A, lane 10, shows the cross-linking products obtained with the active fractions from the S-Sepharose columns. The only major cross-link observed is at 70 kDa, indicating a 50-kDa cross-linking partner. The faint band at ~120 kDa is most probable a double cross-link to this protein. The active fraction from the S-Sepharose chromatography analyzed by SDS-PAGE revealed a single band of 50 kDa (Fig. 2A, lane 4). Mass spectrometry of seven peptides obtained from the excised protein from the SDS gel identified the purified protein as eEF1A. The experimental data found are in good agreement with the predicted sizes shown in parentheses (in daltons): 466.2 (466.25), 537.2 (537.29), 652.3 (652.32), 765.4 (765.40), 878.4 (878.48), 979.5 (979.53), and 1078.6 (1078.60). However, the possibility remained that eEF1A, an abundant cytosolic protein, is contaminated by another protein that is the actual cross-linking partner. To rule out this possibility, we purified recombinant eEF1A expressed as a GST fusion protein in *E. coli* (see "Materials and Methods") and assayed it for cross-linking to 169 aa pPL-MN NCs. We found that both GST-eEF1A fusion protein and eEF1A produced by limited thrombin digestion of the fusion protein were able to cross-link to the 169 aa pPL-MN NC (Fig. 2B). These data prove that it is indeed eEF1A that interacts with the RNCs.

**eEF1A Associates with a Wide Range of Nascent Chains**—We next examined whether eEF1A can interact with other NCs of varying lengths and amino acid sequences. Both purified eEF1A and rabbit reticulocyte lysate were assayed for the production of cross-links to the following NCs: 85 and 221 aa β-actin (Fig. 3A), 86 and 169 aa pPL-MN (Fig. 3B), and 77 and 197 aa flLuc (Fig. 3C). Purified eEF1A cross-linked to all NCs tested (Fig. 3, A–C, lanes 2 and 5, arrow). In general, the incubation of RNCs with rabbit reticulocyte lysate also produced the cross-linking product expected of eEF1A (Fig. 3, A–C, lane 6, and C, lane 3, arrow). However, in two cases, 85 aa β-actin (Fig. 3A, lane 3) and 86 aa pPL-MN (Fig. 3B, lane 3), the expected band was faint, but the longer versions of the same NCs 169 aa pPL-MN and 221aa β-actin did cross-link efficiently with eEF1A in the reticulocyte lysate. Given that the shorter NCs cross-linked to purified eEF1A, this finding suggests that other components in the reticulocyte lysate may compete for binding to the NCs and that eEF1A competes...
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better for binding to the longer NCs. In agreement with this hypothesis, rabbit reticulocyte lysate (RRL) incubated with NCs clearly yields other cross-linked products such as αNAC for 85 aa β-actin (Fig. 3A, lane 3), p120 for 86 aa pPL-MN (Fig. 3B, lane 3), and both NAC subunits and p120 for fLuc (Fig. 3C, lane 3). In some cases, the decreased intensity of the cross-link to eEF1A for the shorter NC may be because of fewer lysines available for cross-linking. For example, 85 aa β-actin has only five lysines compared with 10 lysines in 221 aa β-actin (see Table I). However, this is not the case for pPL-MN, which has only two lysines at positions 4 and 9, the same for 86 aa pPL-MN as for 169 aa pPL-MN. This finding suggests that eEF1A may be more effective than other factors at binding to the distal end of the NC where it is no longer associated with the ribosome. An extension of this reasoning is to ask whether eEF1A can bind to newly synthesized polypeptides even after their release from the ribosome.

eEF1A Associates with Unfolded Polypeptide Chains Released from the Ribosome—To test this idea, high salt-stripped 133 aa β-actin RNCs were incubated with purified eEF1A or rabbit reticulocyte lysate, depleted of ribosomes. Puromycin and RNase A were then added to release the NCs from the ribosome. After centrifugation through a high salt-sucrose cushion, the ribosomes with bound NCs were obtained in the pellet, and the released NCs together with the RAFs were recovered from the supernatant. Both fractions were collected and then irradiated in parallel. Without puromycin or RNase A, very few NCs were released from the ribosome, and consequently, neither β-actin nor a cross-linked band was detected in the supernatant fraction (Fig. 4, lanes 4 and 8). In the presence of puromycin and RNase A, a larger fraction of NCs was released from the ribosome, and a band of puromycin-released 133 aa β-actin cross-linked to eEF1A was detected in the supernatant fraction (Fig. 4, lanes 5 and 9). Note that more than half of the released 133 aa β-actin polypeptide cross-links to eEF1A regardless of whether purified protein (Fig. 4, lane 5) or reticulocyte lysate (Fig. 4, lane 9) was used. These results indicate that eEF1A still associates with polypeptide chains even after their release from the ribosome. Similar results were obtained when the NCs were first released and then eEF1A was added (data not shown).

It has been shown previously that most RAFs interacting with NCs do so only in the context of the ribosome. The experiments described above for Fig. 4 indicate that eEF1A is in this way different and confirm that other RAFs do not cross-link to released polypeptides. NCs cross-link to a variety of RAFs including eEF1A and α- and β-NAC and p120 (Fig. 4, compare lanes 1 and 6). As expected, the other RAFs such as NAC and p120 are not seen cross-linking to puromycin-released β-actin (Fig. 4, lane 9), consistent with previous findings (20).

The experiments described above establish that eEF1A can bind both to NCs and to released polypeptides, but they shed no light on the possible function of eEF1A in this context. We next tested the time dependence of the interaction using full-length transcripts encoding two different proteins, fLuc and pPL-M (Fig. 5), to further explore the interaction of eEF1A with polypeptides. pPL-M harbors three-point mutations in the signal peptide of pPL, which prevents the protein from interacting with SRP, and consequently blocks its secretion from cells. During its folding in the lumen of the ER, prolactin forms three disulfide bridges, which cannot be formed in the cytosolic environment. This is one reason why pPL-M cannot fold correctly.
eEF1A Mediates the Refolding of Firefly Luciferase—The ability to associate with unfolded polypeptide chains after their release from the ribosome is reminiscent of chaperones, a class of proteins that have refolding activity when assayed on denatured proteins. In addition, it has been established that the prokaryotic homologue of eEF1A has chaperone-like activity (33, 34). This finding, together with our observation that eEF1A only interacts with proteins that are unable to fold in the cytosolic environment, suggests that eEF1A may be able to refold proteins. To test this hypothesis, we asked whether eEF1A could mediate the refolding of chemically denatured ffLuc. ffLuc was denatured in the presence of 6 M guanidinium-HCl and allowed to refold upon dilution of the denaturant in the absence or presence of purified eEF1A. Refolding was followed by assaying the luciferase enzymatic activity (see "Materials and Methods"). A time course shown in Fig 6A demonstrated that maximum refolding is complete by 20–30 min. In this experiment, both eEF1A and the GST-eEF1A fusion protein at 130 nM were able to restore ~30% native ffLuc activity compared with ~5% spontaneous refolding on dilution into buffer alone or buffer containing 200 nM GST. Refolding was also concentration-dependent with as little as 10 nM eEF1A restoring 12% ffLuc activity. Maximum activity of ~35% control required 150–180 nM eEF1A. This concentration of eEF1A is present in 5% rabbit reticulocyte lysate (100% rabbit reticulocyte lysate is ~2.5 μM eEF1A), and indeed 150 nM eEF1A, 180 nM GST-eEF1A, and 5% RRL all produce a comparable degree of refolding of ffLuc at ~35% (Fig. 6C). Although this is the maximum degree of refolding seen with eEF1A at any concentration, increased concentrations of RRL (up to 30%) restore additional ffLuc activity to a maximum of 80%. As a negative control, 150 nM IgG, an irrelevant protein, did not significantly restore ffLuc activity. Because most chaperones are ATP-dependent, we next tested whether eEF1A-stimulated refolding of ffLuc requires a source of energy. Refolding in the presence of GST-eEF1A was not affected by the addition of GTP, GDP, GTPγS, ATP, or apyrase (Fig. 6D and data not shown). Taken together, these results show that eEF1A has chaperone-like activity.

under the conditions provided by the reticulocyte translation system. In contrast, ffLuc, a peroxisomal protein, is able to fold in reticulocyte lysate as demonstrated by its acquisition of enzymatic activity (30).

Synchronized translations were sampled at intervals up to 44 min (including a 3-min initiation time, see "Materials and Methods") and assayed for cross-links to translated proteins released by normal termination from the ribosome. Fig. 5A shows that full-length ffLuc, readily apparent in the supernatant starting from the 14-min time point, shows no major cross-linking product. This is also the time when enzymatic activity is first observed (30). In contrast, full-length pPL-M, first detected in the supernatant at the 7-min time point, displays a major cross-linking band of ~70 kDa, indicating the association of pPL-M with eEF1A (Fig. 5B, asterisk). This experiment demonstrates that eEF1A interacts with the unfolded pPL-M protein but not with the correctly folded ffLuc.

Fig. 3. Association of purified eEF1A with nascent chains. A, 85 or 221 aa β-actin RNCs were high salt-stripped and then incubated with buffer A (lanes 1 and 4), purified eEF1A at a concentration of 160 nM (lane 2 and 5), or 2 μl of rabbit reticulocyte lysate in 18 μl total assay (lane 3 and 6) prior to UV irradiation. B, 86 and 169 aa pPL-MN RNCs were analyzed as described above. C, 77 and 197 aa ffLuc RNCs were analyzed as above. Arrows indicate the position of eEF1A cross-linked to NC, whereas "α" and "β" mark the cross-links to both subunits of NAC and "p" marks the cross-links to p120. Molecular masses in kDa are indicated on the left.

Fig. 4. Association of purified eEF1A to polypeptide chains released from the ribosome. 133 aa β-actin RNCs were high salt-stripped and then incubated with buffer A (lane 1), 160 nM purified eEF1A (lanes 2–5), or 2 μl of RRL in 18 μl total assay (lanes 6–9) for 5 min at 26 °C. Puromycin and RNase A were added as indicated, and then ribosomes and released polypeptide chains were obtained as pellet (p) and supernatant (s), respectively, after centrifugation (see "Materials and Methods") and subjected to UV irradiation. Molecular masses in kDa are indicated on the left.

Fig. 5. eEF1A binds to pPL-M but not to ffLuc after their release from the ribosome. Synchronized translations of ffLuc (A) or pPL-M (B) were stopped by the addition of cycloheximide to a final concentration of 5 mM at time points indicated on top of the gel. After UV irradiation or no irradiation (lane 12 in A and lane 10 in B), RNCs were removed by sedimentation (see "Materials and Methods"), and the supernatants were analyzed by 10% SDS-PAGE and autoradiography. Asterisk indicates the position of eEF1A cross-linked to pPL-M. Molecular masses in kDa are indicated on the left.

FIG. 4. Association of purified eEF1A to polypeptide chains released from the ribosome. 133 aa β-actin RNCs were high salt-stripped and then incubated with buffer A (lane 1), 160 nM purified eEF1A (lanes 2–5), or 2 μl of RRL in 18 μl total assay (lanes 6–9) for 5 min at 26 °C. Puromycin and RNase A were added as indicated, and then ribosomes and released polypeptide chains were obtained as pellet (p) and supernatant (s), respectively, after centrifugation (see "Materials and Methods") and subjected to UV irradiation. Molecular masses in kDa are indicated on the left.

FIG. 5. eEF1A binds to pPL-M but not to ffLuc after their release from the ribosome. Synchronized translations of ffLuc (A) or pPL-M (B) were stopped by the addition of cycloheximide to a final concentration of 5 mM at time points indicated on top of the gel. After UV irradiation or no irradiation (lane 12 in A and lane 10 in B), RNCs were removed by sedimentation (see "Materials and Methods"), and the supernatants were analyzed by 10% SDS-PAGE and autoradiography. Asterisk indicates the position of eEF1A cross-linked to pPL-M. Molecular masses in kDa are indicated on the left.
**DISCUSSION**

Macromolecular crowding in the cytosolic environment necessitates protection of the growing NC as it is translated on the ribosome (2, 4). In addition, some NCs require specific covalent modifications and/or targeting to an organelle such as endoplasmic reticulum, mitochondria, chloroplast, peroxisomes, or the nucleus (3). Finally, although translation is remarkably error-free (one mistake in 10,000 aa (35)), rigorous quality control at the co-translational and post-translational stages is essential to remove protein that for whatever reason cannot attain its functional state. In fact, it has recently been found that in eukaryotic cells, up to 50% of newly translated proteins or rabbit reticulocyte lysate. Enzyme activity was measured after 60 min and expressed as a percentage of the native enzyme control. C, refolding buffer contained no additional protein (buffer) or the indicated concentrations of purified proteins or rabbit reticulocyte lysate. Enzyme activity was measured and reported as described above. Similar results were obtained at a concentration of 20 nM GST-eEF1A.

In this report, we identify and characterize a 50-kDa protein that cross-links to a wide variety of NCs as eEF1A, a elongation factor with a well known function in translation, i.e. delivering aminoacyl-tRNAs to the A-site at the ribosome. This is the first report that eEF1A binds to NCs and additionally to released polypeptides. We have demonstrated the latter point in two distinct ways, a functional assay (i.e. refolding of a denatured protein) and an assay of direct physical association (i.e. cross-linking). Although eEF1A was found to bind to a variety of different NCs (Fig. 3), its binding to released proteins was more selective. As assayed by cross-linking, eEF1A binds to pPL-M (Fig. 5B), a protein that cannot fold correctly under the conditions of the in vitro translation system but not to ffLuc (Fig. 5A), a cytosolic protein that does fold correctly. However, eEF1A is in contact with ffLuc NCs (see Fig. 3), and it can be inferred from eEF1A stimulation of ffLuc refolding (Fig. 6) that it also binds to unfolded ffLuc. In addition, a truncated β-actin released from the ribosome with puromycin cross-links to eEF1A (Fig. 4). Taken together, this data suggest that eEF1A binds primarily to unfolded or not completely folded proteins. A released polypeptide has previously been observed to cross-link to a 50-kDa protein, but the identity of the protein was not determined. In this study, Plath and Rapoport (36) performed cross-linking experiments using yeast pre-pro-α-factor-translated in a rabbit reticulocyte lysate supplemented with yeast microsomes. Pre-pro-α-factor probably does not fold efficiently in the cytosol, because it is translocated into microsomes posttranslationally. If the cross-linked 50-kDa protein is indeed eEF1A, their result is then in agreement with our proposal that eEF1A binds to unfolded proteins.

It is worth noting that the binding of eEF1A to NCs is clearly at a site distal from the peptidyltransferase center in which eEF1A is known to deliver charged tRNAs necessary for the elongation of the NC. The experimental basis for this is as follows. 1) eEF1A cross-links to both 56 aa and 169 aa pPL-MN, which have lysines for cross-linking only at positions 4 and 9, over 76 aa away from the peptidyltransferase center (Figs. 1 and 3B). 2) eEF1A cross-links more efficiently to longer than 76 aa away from the peptidyltransferase center (Figs. 1 and 3B). 3) Full-length pPL-M naturally released from the ribosome also cross-links to eEF1A (Fig. 5B). These results point to a novel function for eEF1A that is distinct from the delivery of aminoacyl-tRNA.

It has been shown before that EF-Tu, the bacterial homologue of eEF1A, has chaperone-like activity (33, 34). We demonstrate here that eEF1A also has chaperone-like activity, and that half-maximal refolding of denatured ffLuc occurs at a molar ratio of ~1:2 of substrate to eEF1A (Fig. 6B). This finding compares well with ratios of 1:1 to 1:10 discovered for rhodanese to EF-Tu or citrate synthase to EF-Tu, respectively. In contrast, HSP70 acts at a ratio of 1:50 (37).
eEF1A and EF-Tu both bind GTP, which must be hydrolyzed for bound aminoacyl-tRNA to be released at the ribosome. An exchange of the bound GDP for a new molecule of GTP requires an accessory protein, eEF1Bα for the eukaryotic system and EF-Ts for prokaryotes. Our studies of eEF1A-mediated refolding could not detect either a requirement for or inhibition by nucleotides, nucleotide analogues, or apyrase treatment. However, these findings for EF-Tu-assisted refolding are controversial. Caldas et al. (33) report that the GDP-bound form of EF-Tu is active in catalyzing refolding of citrate synthase and the GTP-bound form is inactive, although it is not clear from their data whether refolding requires GTP hydrolysis. In contrast, Kudlicki et al. (34) find increased refolding of rhodanese in the presence of GTP and inhibition by GDP or a non-hydrolyzable GTP analogue. They further show that EF-Tu-assisted refolding is greatly stimulated by EF-Ts in the presence of GTP and inhibition by GDP or a non-hydrolyzable GTP analogue. It is possible that in our experiments GTP and GDP had no effect on ffLuc refolding, because all of the eEF1A used was already bound to nucleotide and our apyrase treatment was incomplete. It would then be necessary to supplement the reaction with eEF1Bα to fully resolve this question. However, we think that it is not very likely that chaperone activity is a major function of either EF-Tu in bacteria or of eEF1A in eukaryotes. This is emphasized for eEF1A by our finding that it stimulates a maximum refolding of ffLuc of only ~30% compared with 60% obtained with rabbit reticulocyte lysate (Fig. 6C). In agreement with this finding, studies by Frydman et al. (38) demonstrate that the majority of chaperone activity in reticulocyte lysate is because of HSP70. In the context of this study, the significance of eEF1A-assisted refolding of a denatured protein is its demonstration that eEF1A binds to unfolded protein, and we have not focused on the energy requirement of the process.

It is well recognized that post-translational triage of unfolded or damaged protein can result in rescue by chaperones or in targeting for degradation by proteases (39). Previous studies have shown that eEF1A could stimulate ubiquitin-dependent proteolysis of histone H2A, actin, and α-crystalline (23). In general, the signals that trigger ubiquitination and/or protease degradation of newly synthesized proteins are not known. Here, we propose a model in which eEF1A may function as a key component of such a quality control mechanism. We suggest that once eEF1A has released its cargo of charged tRNA, it is free to bind to the growing NC. Its binding can be successfully competed by other RAFs including NAC, SRP, and HSP70/40. The high concentration of cytosolic eEF1A especially close to the ribosome would ensure that a permanent scanning of the NC is possible and eEF1A binding would then hasten the degradation of these proteins. We will test this hypothesis in future experiments.

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Interaction of the Eukaryotic Elongation Factor 1A with Newly Synthesized Polypeptides

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