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A sequestered pool of aminoacyl-tRNA in mammalian cells

(channeling/compartmentation/protein synthesis)

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ABSTRACT **We have recently proposed that aminoacyl-tRNA is channeled during protein synthesis *in vivo*—i.e., it is directly transferred among the components of the protein-synthesizing machinery and does not mix with aminoacyl-tRNA molecules introduced from outside the cell. To understand the structural basis for these functional properties, we have examined the disposition of aminoacyl-tRNA within the cell. To do this we have developed a Chinese hamster ovary (CHO) permeabilized-cell system using the plant glycoside saponin. We show, using a mixture of free ^{14}C-labeled amino acids and ^{3}H-labeled aminoacyl-tRNAs, that ^{14}C-labeled aminoacyl-tRNAs synthesized endogenously from the free amino acids are preferentially sequestered within the cell, whereas their exogenous ^{3}H counterparts distribute between the inside and outside of the cell based solely on the relative volumes of the two compartments. Furthermore, the endogenous ^{14}C-labeled aminoacyl-tRNA population is resistant to pancreatic ribonuclease action, whereas the ^{3}H molecules are rapidly degraded. We conclude, based on these observations, that aminoacyl-tRNAs synthesized *in vivo* are continually associated with components of the protein synthesis machinery and are thereby retained within the permeabilized cell and are also protected from RNase action. These data provide independent evidence for the channeling model of protein biosynthesis.**

tRNA and its derivatives aminoacyl-tRNA and peptidyl-tRNA play a central role in the process of protein biosynthesis (1). They are the carriers of the monomeric units of proteins and of the elongating polypeptide chains. During protein synthesis these tRNAs interact with a variety of participants in the process including the aminoacyl-tRNA synthetases, the elongation factors, and the ribosome. Understanding how these macromolecular intermediates transfer among the various components of the protein synthetic machinery to sustain the rapid rates of polypeptide chain elongation known to occur *in vivo* is of primary importance for understanding the overall process at the molecular level.

The mammalian protein-synthesizing apparatus is thought to be highly organized within the cell, involving direct interactions among various components and with the cytoskeletal framework of the cell (2–12). Presumably, this organization serves as the structural basis for the efficiency of the protein synthetic process. On the functional side, we have recently presented evidence from studies with electroporated cells that aminoacyl-tRNA is channeled for protein synthesis *in vivo*—i.e., it is directly transferred from aminoacyl-tRNA synthetase to elongation factor to ribosome, precluding its dissociation into the cell fluid where it could mix with exogenously introduced aminoacyl-tRNA (13).

One consequence of the channeling model is that aminoacyl-tRNAs should be continually associated with components of the protein-synthesizing machinery. To test this prediction, we have used saponin-permeabilized Chinese hamster ovary (CHO) cells to examine the disposition of endogenously synthesized aminoacyl-tRNAs within the cell compared with that of the same aminoacyl-tRNAs introduced from the outside. In this paper we show that the two aminoacyl-tRNA populations behave very differently. Whereas the exogenous aminoacyl-tRNAs are freely diffusible and distribute between the intra- and extracellular spaces based solely on their relative volumes, the endogenous aminoacyl-tRNAs remain preferentially associated with the permeabilized cells. Moreover, while the exogenous aminoacyl-tRNAs are sensitive to degradation by ribonuclease, the endogenous population is resistant. These findings indicate that endogenously synthesized aminoacyl-tRNAs are sequestered and protected *in vivo*, and they provide independent support for the channeling model of protein biosynthesis (13–15).

MATERIALS AND METHODS

Materials. Mixtures of 15 ^{14}C-labeled and ^{3}H-labeled amino acids were obtained from ICN and Amersham, respectively. Unlabeled amino acids, bovine pancreas ribonuclease A, creatine phosphate, creatine phosphokinase, GTP, trypan blue, and saponin were purchased from Sigma. Fluoroscein isothiocyanate was obtained from Molecular Probes. Cell culture media were products of GIBCO. Rabbit liver tRNA and ^{3}H-labeled aminoacyl-tRNA ([^{3}H]aminoacyl-tRNA) were prepared as described (13). The aminoacyl-tRNA preparation was treated with sodium periodate to inactivate any uncharged tRNA molecules that remained after charging.

Cell Culture. CHO cells were obtained from J. Pachter of this institution. They were maintained as monolayers in Dulbecco's modified Eagle's medium supplemented with 5% (vol/vol) calf serum, 5% (vol/vol) fetal bovine serum, 2 mM glutamine, and 0.35 mM proline. Cells were cultured at 37°C in air/5% CO_2 in Falcon flasks (75 cm^2) and transferred every 3–4 days. Approximate cell numbers were determined in a hemacytometer.

Permeabilization of Cells. Confluent cells were washed once (≈5 ml per flask) with Dulbecco's phosphate-buffered saline (PBS) (137 mM NaCl/5 mM KCl/1.4 mM KH_2PO_4/8 mM Na_2HPO_4) and once with solution S [140 mM KCl/10 mM NaCl/2.5 mM $Mg(C_2H_3O_2)$/0.1 μM $CaCl_2$/20 mM Hepes, pH 7.7]. Four milliliters of solution S containing saponin at 30 μg/ml was added to each flask, followed by incubation for 5 min at 37°C. After removal of the saponin solution, 4 ml of solution S was added, the flasks were placed on ice, and cells were harvested with a GIBCO cell scraper. The contents of two flasks (≈5 × 10^7 cells) were centrifuged

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Abbreviations: [^{14}C]amino acid, ^{14}C-labeled amino acid. According to International Union of Biochemistry nomenclature, usually followed by this journal, the correct nomenclature for the ^{3}H-labeled and ^{14}C-labeled aminoacyl-tRNAs is amino[^{3}H]acyl-tRNA and amino[^{14}C]acyl-tRNA rather than [^{3}H]aminoacyl-tRNA and [^{14}C]aminoacyl-tRNA, respectively.

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and resuspended in 0.25 ml of PS buffer [60 mM KCl/5 mM $Mg(C_2H_3O_2)$/10 mM Tris·HCl, pH 7.5/6 mM dithiothreitol]. A small portion of cell suspension was taken at this time for determination of the efficacy of the permeabilization procedure by measurement of trypan blue entry into cells. Permeabilized cells were used immediately for experiments.

Measurement of Aminoacylation, Aminoacyl-tRNA Distribution, and Protein Synthesis. The total volume of cell suspension was determined with a pipet by using a microtip that was cut to give a larger opening. One-half volume of a solution containing 1 mM ATP, 0.25 mM GTP, 10 mM creatine phosphate, creatine phosphokinase at 125 μg/ml, 0.4 mM ^{14}C-labeled amino acid ([^{14}C]amino acid) mixture (15 of 20 labeled), and/or 270 pmol of [^{3}H]aminoacyl-tRNA mixture (15 of 20 labeled, and based on average specific radioactivity) was added. The cell suspension was gently mixed, and 40- to 45-μl aliquots were added to microcentrifuge tubes; zero time samples were taken at this point. The remaining tubes were incubated for various times at 27°C. At each time point two tubes were taken and centrifuged; the supernatant fraction (representing material outside the cell) and the resuspended cell fraction in PBS (representing material inside the cell) from each were precipitated with 10% trichloroacetic acid. One of the two samples was boiled for 5 min to destroy aminoacyl-tRNA. The precipitates from both samples were collected and counted as described (13). The radioactivity resistant to boiling in trichloroacetic acid was considered to represent protein synthesis, whereas the hot acid-sensitive material (difference between total and hot acid-resistant radioactivity) was considered to represent aminoacyl-tRNA.

Measurement of Fluorescent Macromolecule Entry into Permeabilized Cells. Rabbit liver tRNA and bovine pancreas ribonuclease A were labeled with fluorescein isothiocyanate as described by Plumbridge *et al.* (16) and Maeda *et al.* (17). The derivatized tRNA sample was incubated with permeabilized CHO cells for ≈5 min at room temperature, and the derivatized RNase A was incubated as described in the legend to Fig. 4. Fluorescently labeled cells were analyzed live or after fixation with 3.7% formaldehyde by using a Bio-Rad MRC-600 confocal microscope.

RESULTS

Properties of Saponin-Permeabilized CHO Cells. The plant glycoside saponin has been used as a mild permeabilizing agent for a variety of mammalian cell types, including CHO cells (e.g., refs. 18–20). Saponin has the advantage over many other permeabilizing agents in that at lower concentrations the plasma membrane is affected primarily, whereas other internal membranes remain intact (18–20). For our studies we used a low concentration of saponin, 30 μg/ml, and treatment of cells for 5 min at 37°C to try to minimize cell damage. Under these conditions all cells were rendered permeable, as judged by the entry of trypan blue. Of more importance, these conditions also completely permeabilized the cells to tRNA and RNase A, as shown by the presence of fluorescent derivatives of these molecules throughout the treated cells, visualized with a confocal microscope. The microscopic observations also showed that the permeabilized cells were morphologically unchanged despite the alterations in the plasma membrane, in agreement with other studies (18, 19).

The permeabilized-cell population retained the ability to incorporate amino acids into protein at a rate amounting to ≈15% that of intact cells (Fig. 1). This rate is much higher than usually found in studies of eukaryotic *in vitro* protein synthesis (21, 22), even though little was done to optimize conditions, indicating that a significant amount of the protein-synthesizing system components remains within the cells after permeabilization, despite the fact that this procedure is known to lead to the release of soluble components (18). Moreover, the high rate of synthesis suggests that this system

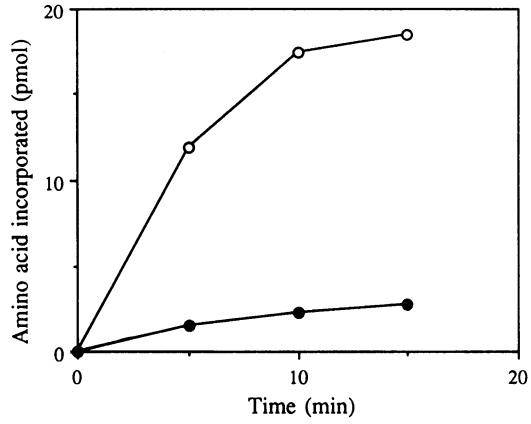

FIG. 1. Comparison of protein synthesis in intact and permeabilized CHO cells. Intact and saponin-permeabilized cells were incubated under identical conditions to measure protein synthesis. At the indicated times, aliquots of 3 × 10^6 cells were taken, precipitated with 10% trichloroacetic acid, and boiled for 5 min to solubilize labeled aminoacyl-tRNA. Precipitates were collected on Whatman GF/C filters and counted. ○, Intact cells; ●, saponin-permeabilized cells.

may retain much of the structural organization of the intact cell. On the other hand, measurement of total RNA (determined by prelabeling with [^{3}H]uridine) in the cell supernatant and cell pellet fractions of the saponin-treated cells indicated that 30–40% of the RNA was released from the cells (data not shown), suggesting that some cell breakage probably occurs during the permeabilization procedure.

Distribution of Endogenous and Exogenous Aminoacyl-tRNAs Between Inside and Outside of Cells. To examine the *in vivo* status of endogenously synthesized aminoacyl-tRNAs, a mixture of ^{14}C-labeled amino acids (15 radioactive) and the corresponding [^{3}H]aminoacyl-tRNAs was incubated with permeabilized cells, and at various times aliquots were withdrawn from the incubation mixture and separated into a cell fraction and a supernatant fraction, representing the inside and outside of the cells, respectively. The ^{14}C-labeled amino acids that had been attached to the endogenous tRNA molecules during the incubation period were quantitated for their distribution between the intra- and extracellular compartments. Distribution of the [^{3}H]aminoacyl-tRNAs in the same samples served as an internal control for the same molecules not synthesized endogenously.

The data in Fig. 2 show that the [^{3}H]aminoacyl-tRNAs and [^{14}C]aminoacyl distributed very differently between the inside and outside compartments. For the [^{3}H]aminoacyl-tRNAs the ratio of inside/outside radioactivity was ≈0.2, exactly as expected from the ratio of the volumes of the two compartments. Moreover, this ratio did not change with the time of incubation, suggesting that the [^{3}H]aminoacyl-tRNAs very rapidly reached their equilibrium distribution. This conclusion was substantiated by varying the cell-to-supernatant fraction ratio (Fig. 3). At each ratio the [^{3}H]aminoacyl-tRNAs distributed according to the relative volumes of each fraction, as shown by the almost 45° slope to the line. Thus, the distribution of the [^{3}H]aminoacyl-tRNAs indicated that exogenous aminoacyl-tRNAs could diffuse freely between the inside and outside of the cell.

However, the [^{14}C]aminoacyl-tRNAs showed a distinctly different pattern, with an initial distribution between the inside and outside of the cell of 2.8 that gradually decreased to ≈2.2 over a period of 35 min. Compared to the distribution of the ^{3}H molecules, these data indicate a preferential retention of the endogenously synthesized aminoacyl-tRNAs of ≈25-fold in this experiment. In various experiments retention of the endogenous molecules and their rate of decline varied

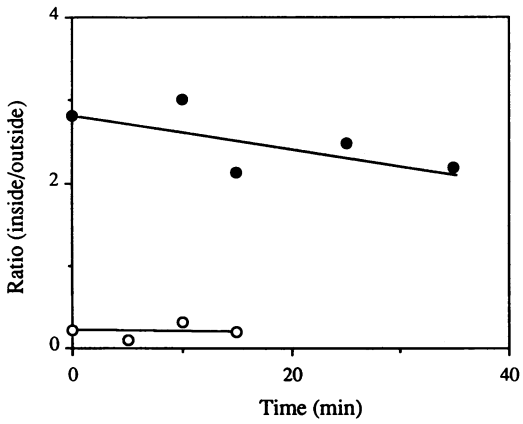

FIG. 2. Distribution of endogenous and exogenous aminoacyl-tRNAs. Permeabilized cells were incubated with mixtures of ^{14}C-labeled amino acids and the corresponding [^{3}H]aminoacyl-tRNAs as described. At the indicated times samples were withdrawn and processed to determine the amount of ^{14}C-labeled aminoacyl- ([^{14}C]aminoacyl-) and [^{3}H]aminoacyl-tRNA associated with the cells and in the supernatant fraction. Data are presented as the ratio of [^{14}C]aminoacyl-tRNA and [^{3}H]aminoacyl-tRNA inside the cell to that found outside. ●, [^{14}C]aminoacyl-tRNA; ○, [^{3}H]aminoacyl-tRNA.

somewhat but never decreased below a 5-fold preferential retention. The variation in different experiments probably reflects different degrees of initial permeabilization and cell damage occurring during the permeabilization procedure and during incubation. In addition, some actual leakage of the endogenous aminoacyl-tRNAs may occur. Nevertheless, these data indicate that a pool of endogenously synthesized aminoacyl-tRNAs is sequestered within cells and is not subject to mixing with the cellular fluid as are their exogenously supplied ^{3}H-labeled counterparts.

RNase Resistance of Endogenous Aminoacyl-tRNA. To ensure that the cell-associated ^{14}C radioactivity that was presumed to be in aminoacyl-tRNAs based on its hot-acid solubility (see *Materials and Methods*) was, in fact, in those compounds, we examined other procedures known to destroy aminoacyl-tRNAs for their ability to eliminate the excess ^{14}C present in the cells. The data in Table 1 show that incubation with KOH, which deacylates aminoacyl-tRNA, solubilized as much radioactive material as the standard hot trichloroacetic acid treatment. The resistant ^{14}C radioactivity in each instance represents newly synthesized protein, which is known to remain acid-precipitable after boiling or alkali treatment. Surprisingly, in contrast to what would have been expected for aminoacyl-tRNA, almost none of the cell-associated ^{14}C radioactivity was solubilized by treatment with pancreatic RNase. This finding raised the possibility that the endogenous aminoacyl-tRNA was somehow protected against RNase action.

Table 1. Effect of various treatments on acid-precipitable radioactivity in permeabilized cells

Treatment	^{14}C-labeled amino acid incorporated, pmol
None	21.2
10% trichloroacetic acid, 5 min, 100°C	10.9
0.5 M KOH, 20 min, 37°C	8.6
RNase A at 10 μg/ml, 10 min, 27°C	20.6

Permeabilized cells were prepared and incubated as described in *Materials and Methods* for 7 min at 27°C, except that only ^{14}C-labeled amino acids were used as precursor. The cell-associated radioactivity was then subjected to the indicated treatments before precipitation and counting.

To examine this point in more detail, permeabilized cells, containing both exogenous [^{3}H]aminoacyl-tRNAs and newly synthesized [^{14}C]aminoacyl-tRNAs from a preincubation, were treated with increased amounts of pancreatic RNase. Fig. 4 shows that the [^{3}H]aminoacyl-tRNAs are readily solubilized by the RNase treatment; however, the ^{14}C counterparts are highly resistant. The resistance of the endogenous [^{14}C]aminoacyl-tRNAs was not due to lack of entry of the RNase because fluorescently labeled RNase readily en-

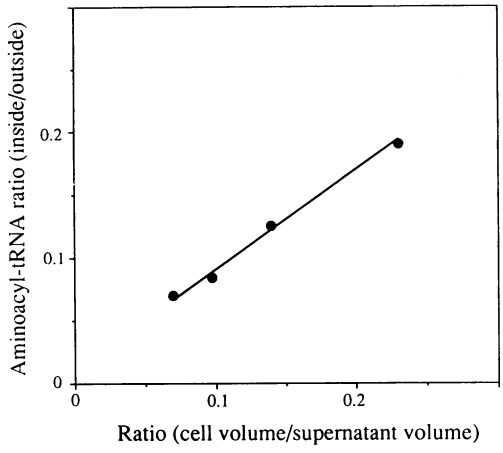

FIG. 3. Distribution of exogenous aminoacyl-tRNA at different ratios of cell to supernatant fraction. Saponin-permeabilized cells (5×10^7) were resuspended in 180 μl of PS buffer. After measurement of the total volume of cell suspension, an additional half volume of the same buffer containing 250 pmol of [^{3}H]aminoacyl-tRNA was added. The final suspension was mixed carefully, distributed to eight microcentrifuge tubes, and incubated 2 min at 27°C. Different amounts of PS buffer were then added to four pairs of tubes to change the ratio of internal to external volume; the suspensions were then incubated for an additional 7 min. Cells were spun down and resuspended in PBS; the cell and supernatant fractions were each precipitated in 10% trichloroacetic acid and processed to quantitate aminoacyl-tRNA. Similar results were obtained when the cells first were diluted, and then aminoacyl-tRNA was added.

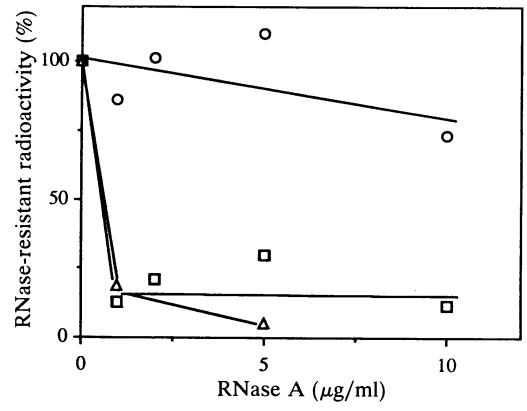

FIG. 4. Effect of RNase on endogenous and exogenous aminoacyl-tRNA in permeabilized cells. Permeabilized cells ($\approx 8 \times 10^6$) were incubated for 7 min with ^{14}C-labeled amino acids and [^{3}H]aminoacyl-tRNAs as described to synthesize endogenous [^{14}C]aminoacyl-tRNAs and then placed in ice. Two samples (for total and hot acid-resistant radioactivity) were used to determine the original amount of [^{14}C]aminoacyl- and [^{3}H]aminoacyl-tRNA; this value was set at 100%. The remaining four samples were incubated for 10 min at 27°C with the indicated concentration of RNase A, centrifuged to isolate the cell fraction, and precipitated with 10% trichloroacetic acid. The total ^{3}H and ^{14}C radioactivity in each sample minus the hot acid-resistant value from the non-RNase-treated sample is presented. In a second experiment cells were incubated with ^{14}C-labeled amino acids, as above, but before RNase treatment the cell fraction was homogenized. □, [^{3}H]aminoacyl-tRNA; ○, [^{14}C]aminoacyl-tRNA; △, [^{14}C]aminoacyl-tRNA, homogenized.

tered permeabilized cells, as noted earlier. Moreover, when cells were homogenized before the RNase treatment, the ^{14}C radioactivity was solubilized as well. These data show, first of all, that the cell-associated ^{14}C radioactivity was, in fact, in aminoacyl-tRNA. More importantly, these results show that the endogenously synthesized aminoacyl-tRNAs are disposed within the cell in a manner that protects them from the action of the RNase, whereas the exogenously supplied ^{3}H molecules are not protected.

Effect of Protein Synthesis on Distribution of Endogenous Aminoacyl-tRNA. To assess whether ongoing protein synthesis is necessary for the relative retention of endogenous aminoacyl-tRNA, permeabilized cells were treated with the protein synthesis inhibitor emetine, and the ratio of ^{14}C radioactivity inside and outside of the cell was determined. At concentrations of emetine up to 150 μM, protein synthesis in both intact cells and saponin-permeabilized cells was inhibited 70–80%. Under these conditions the endogenous [^{14}C]aminoacyl-tRNA population was selectively retained within the permeabilized cell to the same degree as seen in uninhibited cells (data not shown). Thus, ongoing protein synthesis does not appear to be required for the observed distribution of endogenous aminoacyl-tRNAs.

DISCUSSION

The studies presented here support and extend our previous suggestion (13, 14) that protein synthesis is a channeled pathway—i.e., the aminoacyl-tRNA intermediate is directly transferred among the various components of the protein synthetic apparatus and does not dissociate into the cell fluid. Such a model would predict that since endogenously synthesized aminoacyl-tRNAs remain bound, they would not readily diffuse out of permeabilized cells; that was what was found. Moreover, the endogenous aminoacyl-tRNAs were protected against RNase action, supporting the idea that they are continually associated with components that protect them from degradation. This behavior contrasts dramatically with that of exogenously supplied aminoacyl-tRNAs, which rapidly equilibrate between the inside and outside of the cell and which are readily degraded by RNase treatment. Thus, these data indicate that the endogenously synthesized and exogenously supplied aminoacyl-tRNA populations do not freely mix with each other, supporting our contention (13) that the protein-synthesizing system exists as a closed unit to which only free amino acids can enter.

This result does not mean that the protein-synthesizing system is necessarily a *physically* separate compartment, but only that it is *functionally* separate due to a direct transfer mechanism, operating in such a way that intermediates cannot enter the pathway. Still, a channeled pathway might be expected to display some structural organization of its various components, and as noted in the introduction, this is, indeed, the case. It is becoming increasingly clear that the cytoskeletal framework of the cell plays a role in this organization (see, e.g., refs. 11 and 12), and the hydrophobic character and lipid-binding properties of some of the components (23) suggest that membranes are involved as well. Obviously, the associations observed *in vitro* after cell disruption represent only the most stable interactions, and weak interactions presumably are destroyed upon preparation of cell extracts. Studies such as these with permeabilized cells may preserve a greater degree of organization and result in a closer approximation of the situation *in vivo*. However, additional work with a variety of permeabilized-cell systems to obtain one that is minimally damaged would clearly be helpful in this regard.

Although the saponin-permeabilized system has been extremely useful as a first attempt for providing insights about the protein-synthesizing system *in vivo*, it definitely can be improved upon. Thus, although a pool of aminoacyl-tRNA is clearly sequestered within these cells, we still find that about one-third to one-half of the total aminoacyl-tRNA population does leak out. It has not been possible to conclusively establish whether this leakage represents unbound molecules within the cell or cells and internal structure damaged by the permeabilization procedure. Because a similar amount of total RNA also leaks out, we suspect that damage to the cells is responsible.

Despite these reservations, the permeabilized-cell system has been a useful complement to our earlier work with electroporated cells (13) because it can be manipulated much more easily and can be used to examine structural questions, such as those posed here. Taken together, the two different approaches provide a strong argument for the channeling hypothesis of protein synthesis. Further work on the intracellular location, structural organization, assembly of the components, and the high efficiency of the process using microscopic, chemical cross-linking, and enzymatic procedures will be needed to extend our understanding of the protein-synthesizing machinery *in vivo*.

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