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Biochemistry

Nascent peptide as sole attachment of polysomes to membranes in bacteria

(puromycin reaction/*Escherichia coli*/protein secretion)

WALTER P. SMITH, PHANG-C. TAI, AND BERNARD D. DAVIS

Bacterial Physiology Unit, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT **When membrane–polysome complexes from *Escherichia coli* were treated with puromycin, at various Mg^{2+} and K^+ concentrations, the bulk of the ribosomes were released from the membrane. Moreover, many released ribosomes remained attached to mRNA (pseudopolysomes). These results suggest that ribosomes are attached to the membrane in bacteria solely by their nascent chain. Without a direct attachment the conformational changes associated with chain elongation on the ribosome cannot force the growing chain through the membrane, and so alternative sources of energy for the transfer must be considered.**

Palade and coworkers, observing extensive binding of ribosomes to the endoplasmic reticulum in hepatic and pancreatic cells, suggested that such bound ribosomes secrete proteins across membranes as growing chains (reviewed in ref. 1). Milstein *et al.* (2) provided evidence for the presence of a special leading sequence which might participate (3) in this process: when mRNA from myeloma cells is translated by an *in vitro* system lacking homologous membrane, the immunoglobulin chains appear as larger precursors containing an NH_2-terminal sequence that is absent from the final product. Similar precursors have since been demonstrated for other proteins secreted by animal cells (4, 5), and the additional sequences have been found to contain a preponderance of hydrophobic residues (6). Bacteria have recently been shown, through studies with membrane-free preparations, to form similar precursors of secreted proteins (7, 8).

The suggested extrusion of growing chains across membranes has recently been directly demonstrated in this laboratory (9). When *Escherichia coli* spheroplasts are treated with a reagent that acylates amino groups but cannot penetrate cell membranes (acetyl[^{35}S]methionyl methylphosphate sulfone), a portion of the label becomes fixed to growing polypeptide chains, which can be shown to be still attached to polysomes on the other side of the cytoplasmic membrane (9). In addition, the membrane-associated polysomes isolated from the cell can complete these extracellularly labeled incomplete chains, and a significant fraction of the product has been identified as a major periplasmic protein, alkaline phosphatase.

The hydrophobic NH_2-terminal signal region of the growing chain of a secreted protein presumably is threaded into the membrane shortly after emerging from the ribosome (2, 6). However, the source of energy for the vectorial transport of the rest of the chain is obscure. One possibility would use the energy of chain elongation, through conformational changes in the ribosome, to push the growing chain into a suitable pore in the membrane (10, 11). This mechanism would require a firm attachment of the ribosome, apart from the nascent chain, to a receptor on the inner surface of the membrane. Such an attachment may indeed be present in eukaryotic cells, because after release of the nascent chain by puromycin the ribosomes in the membrane–polysome fraction remain attached to the membrane; they can be released by high concentrations of KCl or by EDTA (12, 13). Moreover, Sabatini and coworkers (11, 14) have demonstrated the retention of two membrane-associated proteins on ribosomes released under appropriate conditions.

This paper reports that in bacteria, in contrast, the ribosomes may be attached to the membrane solely by the nascent chain, because in our purified system puromycin treatment (at various K^+ and Mg^{2+} concentrations) released the polysomes from the membrane. Accordingly, sources of energy for vectorial transport other than those associated with chain elongation must be considered. A preliminary account of the work has appeared (15).

MATERIALS AND METHODS

Bacterial Strains and Growth. *E. coli* K12 CW3747, Met^- and constitutive in the synthesis of alkaline phosphatase, was obtained from the American Type Culture Collection (no. 27259). Cells were grown at 37°, with vigorous aeration (unless otherwise indicated), in minimal medium A (16) supplemented with 0.4% glucose and methionine at 20 μg/ml. Membrane–polysome complexes were prepared as described (9). Briefly, a lysate, prepared by the freeze-thaw-lysozyme method (17), was applied to a discontinuous sucrose gradient of 2 ml 1.0 M sucrose and 3 ml 1.8 M sucrose, each in 10 mM Tris·HCl, pH 8.0/50 mM KCl/10 mM $Mg(OAc)_2$ (buffer A). After centrifugation for 18.5 hr at 42,500 rpm in an SW 50.1 rotor, 2 ml from the interface of the sucrose layers (membrane-associated polysomes) was collected and was concentrated by centrifugation through a second sucrose gradient under the same conditions. Alternatively, the collected membrane–polysome complexes (100 A_{260} units) were pelleted and were further purified by passage through a 60 × 1 cm Sepharose 2B column. Fractions (1 ml) were eluted with buffer A containing 1 mM dithiothreitol.

Labeling of RNA, Lipids, and Nascent Peptide. Cells were grown for two generations with [^{3}H]uracil (25 Ci/mmol, 1 μCi/ml), to label ribosomes, and with [^{14}C]oleic acid (60 mCi/mmol, 0.1 μCi/ml), to label membranes. To label nascent peptides, cells were pulsed with a mixture of 15 ^{14}C-labeled amino acids (40 mCi/mmol, 0.1 μCi/ml) for 20 sec, chloramphenicol (200 μg/ml) was added, and the culture was poured over ice.

***In Vitro* Puromycin Reaction.** Membrane-associated

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Abbreviations: EF-G, prokaryotic elongation factor G; Buffer A, 10 mM Tris·HCl, pH 8.0/50 mM KCl/10 mM $Mg(OAc)_2$.

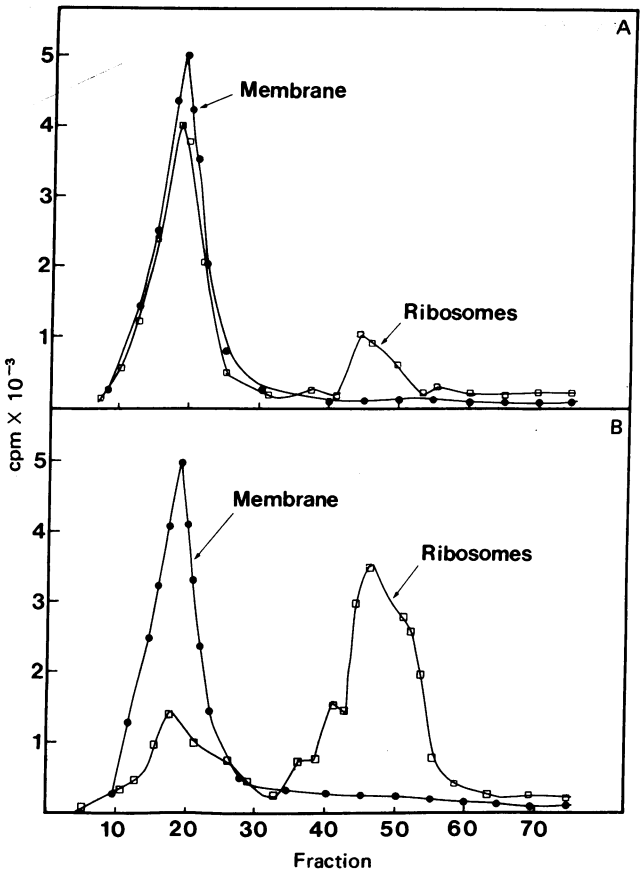

FIG. 1. Puromycin treatment of membrane–polysome complexes. Complexes containing membrane labeled with [^{14}C]oleic acid (●) and ribosomes labeled with [^{3}H]uracil (□) were incubated at 37° for 15 min with (*B*) and without (*A*) puromycin, EF-G, and GTP. The mixtures were analyzed on a Sepharose 2B column.

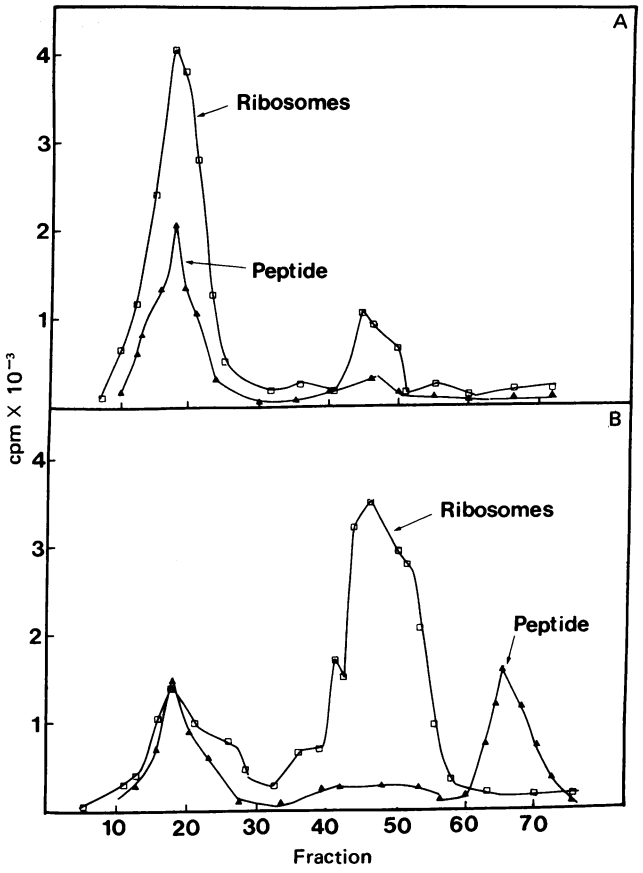

FIG. 2. Puromycin treatment of membrane–polysome complexes. Same as in Fig. 1 except that the membrane was not labeled and the growing chains were pulsed for 20 sec with ^{14}C-labeled amino acids (▲).

polysomes (1 A_{260} unit), labeled either with [^{3}H]uracil and [^{14}C]oleic acid or with [^{3}H]uracil and ^{14}C-labeled amino acids as described above, were incubated in a 100-μl reaction mixture containing buffer A, 1 mM dithiothreitol, 50 μg of puromycin, 50 μg of GTP, and 9 μg of prokaryotic elongation factor G (EF-G). After incubation the samples were cooled quickly to 0° and applied to a Sepharose 2B (20 × 1 cm) column equilibrated with Tris·HCl buffer containing $Mg(OAc)_2$ and KCl at the concentrations in the reaction mixture. The column was eluted with the same buffer, and 0.25-ml fractions were collected. Ten milliliters of a Triton/xylene scintillation fluid was added and radioactivity was measured in a Searle Isocap 300 counter.

Reagents. [^{14}C]Oleic acid, [^{3}H]uracil, and the ^{14}C-labeled amino acid mixture were obtained from New England Nuclear Corp. All other chemicals were of reagent grade. EF-G was purified to homogeneity according to published procedures (18).

RESULTS

To determine whether or not membrane-associated bacterial ribosomes remain bound after their nascent chain has been released, membrane-ribosome complexes, with differentially labeled ribosomes and membrane, were incubated with puromycin (along with EF-G and GTP) for 15 min at 37° and the extent of ribosome release was measured. In the initial experiments, the membrane-bound and the freed ribosomes were separated by discontinuous sucrose gradient centrifugation. A large (often >75%) but variable release was observed.

Because the centrifugation produces pressures high enough to have marked conformational effects on ribosomes, which can shift the equilibrium of their dissociation into subunits (19, 20), these effects might possibly also disrupt any weak interactions between membrane and ribosomes. Accordingly, subsequent analyses were all carried out by gel filtration through a Sepharose 2B column, which cleanly and gently separated membrane-associated polysomes from free (cytoplasmic) polysomes and ribosomes (data not shown). This procedure gave the same result, illustrated in Fig. 1. Puromycin released more than 75% of the labeled ribosomes (fractions 40–55) from the labeled membrane (fractions 10–25), whereas control complexes, incubated without puromycin, released only about 15%.

The release of ribosomes from membrane by puromycin in this experiment did not depend on their release from mRNA, for the purified system used lacked the ribosome release factor, a cytoplasmic protein that is required for the release of ribosomes from mRNA after loss of their nascent chain (refs. 21–23; unpublished data). In fact, when analyzed in a continuous sucrose gradient 40% of the released ribosomes sedimented as "pseudopolysomes" [i.e., polysomes without nascent chains (21)], compared to 50% polysomes observed when the ribosomes were separated from the membranes by deoxycholate rather than by puromycin (data not shown).

To throw further light on the connections between polysomes and membrane, a similar experiment was performed with preparations containing pulse-labeled nascent chains. As Fig. 2 shows, after puromycin treatment approximately 50% of the labeled peptide remained associated with the membrane (fractions 10–25). This material should include three classes:

Table 1. Ribosome release at various salt concentrations

Addition	Salt concentration, mM		Ribosomes released from membrane, %
	Mg^{2+}	K^+	
None	10	50	13 ± 12
None	40	50	16 ± 11
Puromycin	10	50	73 ± 24
Puromycin	10	500	81 ± 17
Puromycin	20	50	74 ± 18
Puromycin	40	50	71 ± 22
Puromycin	40	0	68 ± 22

The membrane–polysome fraction was incubated at 37° for 15 min in 10 mM Tris·HCl, pH 8.0, with KCl and $Mg(OAc)_2$ concentrations varied, and with puromycin (together with EF-G and GTP) added as noted. After incubation the samples were analyzed on a Sepharose 2B column, the eluting buffer containing the same concentrations of salts present in the sample. Data presented are the mean (and the range) from six separate experiments.

membrane proteins (completed or incomplete) that remained in the membrane after release by puromycin; peptides released into the interior of vesicles; and peptides on those ribosomes that failed to react with puromycin. Most of the retained labeled peptides fell into the first two classes, for treatment with deoxycholate (following puromycin) left only 10–15% of the total ^{14}C-labeled peptides of the initial membrane–polysomes complexes with the membrane-derived polysomes (data not shown).

This fraction, representing unreacted ribosomes, is somewhat less than the fraction of ribosomes remaining with the membrane after puromycin treatment (25%). However, such a difference would be expected, if some ribosomes (10–15%) remain bound directly to membrane via an unreacted nascent chain and if others remain on the same polysome after loss of that chain. Since ribosomes in lysates generally do not react quantitatively with puromycin, our findings provide no evidence for even a small fraction of ribosomes remaining attached directly to membrane after release of their nascent chain.

In microsomes from animal cells a different response to puromycin has been reported: the ribosomes remain bound to membrane after puromycin treatment under the usual conditions, although they dissociate from the membrane at high concentrations of KCl or with EDTA (12, 13). We therefore tested the effect of puromycin treatment at several concentrations of K^+ and Mg^{2+}, in an effort to detect a residual affinity between ribosomes and membrane in our bacterial system. As Table 1 shows, even when the KCl concentration was decreased to 0 and the Mg^{2+} concentration was increased from 10 to 40 mM, the release of ribosomes from membrane by puromycin was not impaired. It has thus not been possible to demonstrate any attachment of the ribosomes to the membrane except via the nascent chains undergoing extrusion.

DISCUSSION

The data presented show that, when *E. coli* membrane–polysome complexes were treated with puromycin (along with EF-G and GTP), about 75% of the polysomes and 50% of the nascent chains were released from the membrane. Examination of the released material on a sucrose gradient indicated that little polysome degradation occurred, because the complexes, like purified polysomes (21–23), contain little ribosome release factor. It thus appears that mRNA does not contribute significantly toward stabilizing the membrane–polysome complex in bacteria, except via its participation in the formation of nascent chains. The possible role of mRNA in stabilizing membrane–polysome complexes in animal cells is controversial (11, 24–27); it has not been settled because disruption of the membrane–ribosome interaction has involved conditions that also break down polysomes.

The pulse-labeled peptides that remain associated with the membrane fraction after puromycin treatment are probably largely incomplete (and recently completed) membrane proteins that remain embedded in the membrane. In addition, some may be peptides secreted into the interior of membrane vesicles (right side out) after puromycin release. Removal of these components by deoxycholate identified a small fraction (10%–15%) of the total membrane-associated nascent chains as peptides attached to ribosomes that had failed to react with puromycin, perhaps for lack of a required ribosomal or cytoplasmic protein (28).

The proportion of ribosomes remaining associated with the membrane after puromycin treatment (25%) exceeded the proportion of the peptides that remained associated with ribosomes (10%–15%). However, this inequality can be readily explained without postulating a distinct class of ribosomes, with attachments to the membrane other than peptidyl-tRNA. As we have seen, the released polysomes remained largely intact after puromycin treatment, because of the absence of ribosome release factor, and the same was undoubtedly true of the polysomes still attached to membrane. Accordingly, a single membrane-bound peptide that failed to be released by puromycin should link to the membrane not only its own ribosome but others on the same mRNA.

In microsomes from animal cells, after puromycin treatment the ribosomes remain bound to membrane under the usual conditions, but they are released at high concentrations of KCl. In *E. coli*, in contrast, we have found that over a wide range of K^+ and Mg^{2+} concentrations, puromycin releases the polysomes from membrane (except for a fraction that can be accounted for by the usual failure of some ribosomes in lysates to react with puromycin). Furthermore, we have observed similar release of polysomes from membrane by puromycin in membrane–polysome complexes from the Gram-positive *Bacillus subtilis* (unpublished data). These findings suggest that in bacteria ribosomes are bound to membrane solely via the nascent chains being formed on them.

Alternatively, it is conceivable that during protein synthesis the presence of a nascent chain induces a ribosomal conformation that does lead to firm binding to membrane. However, this hypothesis seems unlikely: one might expect high Mg^{2+} or low K^+ concentration to exaggerate the partial binding energy of the ribosome without a nascent chain, just as either exaggerates the nonphysiological binding of other ligands to the ribosome. Hence, even though eukaryotic and prokaryotic cells both use a hydrophobic leading sequence in secreting proteins, it appears that they may differ significantly in the subsequent mechanism of extrusion.

The apparent absence of firm binding of ribosomes to membrane in bacteria, except via the growing chain, has important implications for the mechanism of extrusion. If the bacterial ribosome does not bind directly to the membrane, it cannot act as a fulcrum from which the energy expended in the chain elongation of protein synthesis could be utilized to push a growing peptide through a membrane barrier, because one cannot push a string through a liquid without a surrounding tube. Accordingly, it seems necessary to consider two alternatives: a pulling force, perhaps supplied by spontaneous polypeptide folding at the external surface of the membrane; or a transmembrane channel sufficiently elaborate to transduce metabolic energy into active outward secretion of the chain.

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1. Palade, G. E. (1975) *Science* **189,** 347–358.
2. Milstein, C., Brownlee, G., Harrison, T. & Mathews, M. (1972) *Nature New Biol.* **239,** 117–120.
3. Blobel, G. & Sabatini, D. D. (1971) in *Biomembranes,* ed. Manson, L. A. (Plenum Press, New York), Vol. 2, pp. 193–196.
4. Schechter, I. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2256–2260.
5. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67,** 835–851.
6. Devillers-Thiery, A., Kindt, T., Scheele, G. & Blobel, G. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 5016–5020.
7. Inouye, S., Wang, S., Sekizawa, J., Halegoua, S. & Inouye, M. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1004–1008.
8. Inouye, H. & Beckwith, J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1440–1444.
9. Smith, W. P., Tai, P.-C., Thompson, R. C. & Davis, B. D. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2830–2834.
10. Lampen, J. O. (1974) *Transport at the Cellular Level, Symposia of the Society for Experimental Biology* (Cambridge University Press, Cambridge, England), Vol. 28, pp. 351–374.
11. Sabatini, D. D. & Kreibich, G. (1976) in *The Enzymes of Biological Membranes,* ed. Martonosi, A. (Plenum Press, New York), Vol. 2, pp. 531–579.
12. Adelman, M. R., Sabatini, D. D. & Blobel, G. (1973) *J. Cell Biol.* **56,** 206–229.
13. Rosbash, M. & Penman, S. (1971) *J. Mol. Biol.* **59,** 227–241.
14. Ojakian, G. K., Kreibich, G. & Sabatini, D. D. (1977) *J. Cell Biol.* **72,** 530–551.
15. Smith, W. P., Tai, P.-C., Thompson, R. C. & Davis, B. D. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36,** 897.
16. Davis, B. D. & Mingioli, E. S. (1950) *J. Bacteriol.* **60,** 17–28.
17. Ron, E. Z., Kohler, R. E. & Davis, B. D. (1966) *Science* **153,** 1119–1120.
18. Leder, P. (1971) in *Methods in Enzymology,* eds. Moldave, K. & Grossman, L. (Academic Press, New York), Vol. 20, Part C, pp. 302–306.
19. Infante, A. & Baierlein, R. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 1780–1784.
20. Spirin, S. (1971) *FEBS Lett.,* **14,** 349–353.
21. Tai, P.-C., Wallace, B. J. & Davis, B. D. (1974) *Biochemistry* **13,** 4653–4659.
22. Hirashima, A. & Kaji, A. (1972) *Biochemistry* **11,** 4037–4044.
23. Kung, H. F., Treadwell, B., Spears, C., Tai, P.-C. & Weissbach, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3217–3221.
24. Milcarek, C. & Penman, S. (1974) *J. Mol. Biol.* **89,** 327–338.
25. Lande, M. A., Adesnik, M., Sumida, M., Tashiko, Y. & Sabatini, D. D. (1975) *J. Cell Biol.* **65,** 513–528.
26. Kruppa, J. & Sabatini, D. D. (1977) *J. Cell. Biol.* **74,** 414–427.
27. Grubman, M. J., Weinstein, J. A. & Shafritz, D. A. (1977) *J. Cell Biol.* **74,** 43–57.
28. Glick, B. R. & Ganoza, M. C. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4257–4260.