A 33-kDa polypeptide with homology to the laminin receptor: Component of translation machinery

(polyribosomes/50S cytoplasmic particles/p40)

DOROTHY AUTH AND GEORGE BRAWERMAN*

Department of Biochemistry, Tufts University Health Sciences Campus, Boston, MA 02111

Communicated by David H. Hubel, February 3, 1992

ABSTRACT A 33-kDa polypeptide (termed p40), which shares an antigenic determinant with a laminin receptor and is under translational control, is believed to serve as a precursor to the receptor and to be related to the neoplastic state. The present study of subcellular localization of this protein shows it to be a cytoplasmic component not associated with the plasma membrane. Most of the cellular p40 was found to be associated with polyribosomes as well as with 40S to 60S cytoplasmic particles. Conditions that lead to polysome disruption also caused release of the polysomal form of p40 as smaller particles, and polysome reconstitution was accompanied by uptake of p40 into these structures. Because of the large abundance of this protein in the cells (six to eight copies per ribosome), it is unlikely that it represents a factor that associates with the 40S preinitiation complex. The p40-containing particles appear to represent a newly discovered structure involved in the process of polysome formation.

Previous studies in this laboratory have led to the identification of an abundant mRNA species that is under translational control in a variety of mouse tumor cells (1). The mouse mRNA codes for a polypeptide termed p40 on the basis of its mobility on polyacrylamide gels in the presence of SDS (2, 3). Analysis of a cloned cDNA for the p40 mRNA species showed it to code for a 33-kDa polypeptide (4). A human cDNA clone isolated from a colon carcinoma on the basis of the unusually high abundance of mRNA in the tumor (5) showed nearly complete homology to the mouse p40. A possible relationship between p40 and a laminin-binding protein was suggested by the fact that it contains an octapeptide sequence identical to that of a peptide derived from the 68- to 72-kDa laminin receptor (6). Moreover, a mouse cDNA clone obtained by screening with an antibody to the laminin receptor (7) proved to be essentially identical to the p40 cDNA. A relationship between p40 and the neoplastic state has been proposed (7).

The present study deals with characterization of p40 in the cell. We found no evidence for association of this protein with membranes in cell extracts. Major portions of p40 are associated with 40S to 60S cytoplasmic particles and with polyribosomes in various cell types. Shifts of p40 between the small particle state and polysomes were observed under conditions that lead to generation or loss of polysomes both *in vivo* and in cell extracts. We conclude from our results that p40 is part of a cytoplasmic structure that is involved in the translation process.

MATERIALS AND METHODS

Cells and Lysate Preparation. Mouse sarcoma 180 ascites (S-180) cells were subjected to starvation followed by incu-

bation in complete medium as described (8). Mouse erythroleukemia (MEL) cells (cell line C7D) were cultured as described (3). Cells were collected by centrifugation, washed in 0.9% NaCl, and resuspended in 3 vol of disruption buffer consisting of 100 mM potassium acetate, 20 mM Hepes (pH 7.4), 1 mM MgCl₂, and 2 mM dithiothreitol, and disrupted by passage through a nitrogen pressure cell (Kontes) at 600 psi (1 psi = 6.9 kPa). Centrifugation at 10,000 × *g* for 10 min yielded a pellet consisting of nuclei, membranes, and cell debris, and a supernatant fraction designated as the cytoplasmic extract. The latter was either used directly for the experiments or stored at -70°C after quick freezing. In experiments with polysomes, S-180 cytoplasmic extract was centrifuged for 1 hr at 100,000 × *g* through 2 ml of 20% (wt/vol) sucrose in TKM (50 mM Tris·HCl, pH 7.6/100 mM KCl/1 mM MgCl₂). The preparations contained polysomes as well as free ribosomes and small particles. Rabbit reticulocytes were prepared and lysed as described (9).

Analysis of Centrifugal Fractions. Zone sedimentation was carried out at 4°C either in 10–30% or in 10–40% linear sucrose gradients in 50 mM Tris·HCl, pH 7.6/1 mM MgCl₂/100 mM NaCl or KCl. A 0.5-ml cushion of 2 M sucrose in the same buffer was included at the bottom of the 10–40% gradients. Centrifugation was with a Beckman SW-55 rotor. The distribution of ribosomal material in the gradients was monitored by absorbance at 257 nm. The material in gradient fractions was precipitated overnight by 3 vol of ethanol in the presence of 0.3 M NaCl. The pellets were resuspended in 25 μl of H₂O, and samples were subjected to SDS/12.5% PAGE as described by Laemmli (10). These conditions were somewhat different from those used to identify the p40 translation product (11), and the present relative mobility of p40 (corresponding to that of a 43-kDa polypeptide) differs somewhat from the 40-kDa value estimated previously.

For Western blots, the proteins separated by gel electrophoresis were transferred to nitrocellulose filters in a Transphor apparatus (Hoefer) in 25 mM Tris·HCl/185 mM glycine/20% methanol for 4 h at 400 mA. Residual binding sites on the filters were blocked in 10% bovine serum in phosphate-buffered saline (PBS) for 4 h with gentle shaking. Undiluted culture fluid containing a monoclonal antibody specific for p40 (DOLCE; see ref. 12), was incubated with the blots overnight at 4°C with gentle shaking. The blots were first washed with PBS, and then for 20 min with PBS/10% bovine serum/0.5% Tween 20. For the second antibody incubation, a 1:500 dilution (in PBS) of goat anti-mouse IgM alkaline phosphatase conjugate (Sigma) was applied for 2 h. Subsequent washes included PBS, PBS/10% bovine serum/0.5% Tween 20, 100 mM Tris·HCl (pH 7.4), and 100 mM Tris·HCl/100 mM NaCl/5 mM MgCl₂, pH 9.5 (solution A) for 15 min each. The colorimetric reaction was carried out as described (13) with solution A being substituted for the barbital (Veronal)/acetate buffer. Relative amounts of p40

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: eIF, eukaryotic initiation factor.
*To whom reprint requests should be addressed.

protein were determined by laser densitometry of the stained bands.

RESULTS

Identification and Quantitation of p40 by Immunoblotting.

The experiments in this study were carried out with a monoclonal antibody known to recognize p40 on Western blots (12). Blots of cytoplasmic extracts of MEL cells yielded a major band of ≈ 43 kDa. Extracts of S-180 ascites cells and of mouse 3T3 cells yielded the same 43-kDa band. This material was also abundant in the rabbit reticulocyte lysate (see Figs. 1 and 4).

The identity of the 43-kDa band with the translationally controlled p40 is indicated by various lines of evidence. First, screening of a mouse cDNA library with the monoclonal antibody used in this study yielded a cloned cDNA with the same nucleotide sequence as the p40 cDNA (12). In our hands, cell-free translation of a p40 transcript derived from our own cDNA yielded a labeled polypeptide that comigrated with the band obtained by Western blot (data not shown). The antibody also recognized a minor 43-kDa component in a preparation of eukaryotic initiation factor 4A (eIF-4A) (kindly provided by William Merrick, Case Western Reserve University). Partial amino acid sequencing of the reactive material in eIF-4A preparations showed it to be homologous with the p40 protein (W. Merrick, personal communication).

Western blot analysis of a preparation of eIF-3 (also provided by W. Merrick) yielded a faint band with a mobility corresponding to that of a 38-kDa polypeptide instead of the 43-kDa band (data not shown). The immunoreactive band did not correspond to any of the eIF-3 polypeptides visualized by Coomassie blue staining. It probably represents a breakdown product of p40 that was contaminating the eIF-3 preparation.

Estimates of the amounts of p40 in MEL cells were done with the preparation obtained from W. Merrick used as a reference. The concentration of protein in this preparation, estimated by W. Merrick by quantitation of Coomassie blue staining of the p40 band resolved by gel electrophoresis, was verified by amino acid microanalysis. Comparisons of Western blots indicated the presence of ≈ 70 ng of p40 per μ l of cytoplasmic extract. Comparison with the RNA concentration in the extract, determined as described (14), yielded a value of six to eight copies of p40 per ribosome.

Distribution of p40 in Cell Extracts. Using extracts of MEL cells disrupted mechanically without the use of detergent, p40 was found primarily in the cytoplasmic fraction. Some p40 was present in the crude nuclear pellet but was readily recovered by washing the pellet in disruption buffer. A low-resolution zone centrifugation of the cytoplasmic extract

FIG. 1. Occurrence of p40 as small particles in cytoplasmic extracts. Samples of MEL extract and of reticulocyte lysate, incubated for 5 min at 37°C in the presence or absence of pancreatic RNase (50 μ g/ml), were layered onto a 5-ml 10–40% sucrose gradient and subjected to centrifugation at 50,000 rpm for 45 min. Samples of gradient fractions were used for Western blot analysis as described. Fractions 1 and 7, bottom and top of gradients, respectively. Positions of ribosomal subunits were determined by scanning the gradient for absorbance at 257 nm.

designed to determine whether p40 occurs either as a soluble component or associated with particles showed that much of it occurred as particles sedimenting around 40S to 60S, both in MEL and in rabbit reticulocyte extracts (Fig. 1). Treatment of the MEL extract with the anionic detergent Triton X-100 before centrifugation did not alter its sedimentation characteristics (data not shown).

Some of the p40 was also found to cosediment with heavier particles in the region of the gradient containing polyribosomes (Fig. 1). Destruction of the polysomes by digestion of the extracts with pancreatic RNase led to loss of the polysomal p40 component with apparent release into the small particle fraction (Fig. 1).

A more detailed centrifugal analysis of an extract of rapidly growing MEL cells showed that p40 sediments in a highly heterogeneous fashion with peaks in the ribosomal subunit and in the polyribosome regions (Fig. 2A). Some p40 also seemed to occur as a soluble component. A longer centrifugation provided greater resolution of the slowly migrating components. The data in Fig. 2B show a major peak of p40-containing material around 50S and some heavier heterogeneous material extending into the 60S to 80S region. Fig. 2B also shows the occurrence of a soluble form of p40.

Effects of RNase and EDTA on p40-Containing Particles. The nature of the particles released from polysomes by RNase digestion was examined by comparing the sedimentation behavior of p40-containing material in samples of nuclease-treated and untreated S-180 extracts (Fig. 3). As expected, RNase treatment caused the appearance of 80S

FIG. 2. Sedimentation analysis of p40-containing particles in cytoplasmic extract of MEL cells. Extracts from rapidly growing cells were subjected to zone centrifugation through a 5-ml 10–30% sucrose gradient at 49,000 rpm for either 45 min (A) or 100 min (B). p40 in gradient fractions was analyzed as described in Fig. 1. Quantitative values from Western blots are represented by bar graphs. Continuous lines represent absorbance at 257 nm. For other details, see Fig. 1.

FIG. 3. Sedimentation characteristics of p40-containing particles released from RNase-treated polysomes. Samples of S-180 cytoplasmic extract, incubated in the presence (B) or absence (A) of pancreatic RNase as described in Fig. 1, were centrifuged through a 10–30% gradient at 49,000 rpm for 100 min. Fraction 1, pelleted p40-containing material. See Fig. 2 for other details.

ribosome particles derived from fragmented polysomes (compare Fig. 3 A and B). The untreated S-180 extract contained small p40-bearing particles sedimenting primarily in the 40S to 60S range (Fig. 3A). The RNase-treated extract had, in addition, heavier particles sedimenting mostly around 80S to 100S (Fig. 3B). The 40S to 60S particles normally present in the extracts seemed unaffected by RNase treatment. These results show that the p40-containing structures released from fragmented polysomes are larger and presumably more complex than those represented by the 40S to 60S particles normally present in the extracts.

The structures that carry p40 were found to be sensitive to the Mg-complexing agent EDTA. Ultracentrifugation of preparations of polysomes and small particles incubated for 5 min at 37°C in the presence of 20 mM EDTA caused release of p40 as soluble material remaining at the top of the gradient (data not shown). The ribosomes in the preparations were converted to 50S and 30S particles by this treatment.

Shifts in p40 Distribution in the Reticulocyte Lysate During Translation. p40 occurs primarily in polysomes and in a heterogeneous population of smaller particles sedimenting mainly around 40S to 60S in the unincubated reticulocyte lysate (Fig. 4). A substantial amount of soluble p40 is also present. Incubation for 15 min under conditions that promote

protein synthesis resulted in nearly complete loss of the polysomes, apparently because of limited initiation capacity in the lysate. This was accompanied by loss of the polysomal form of p40 and by a large increase in the soluble form. Inclusion of cycloheximide in the incubation mixture prevented loss of the polysomes as well as of the polysomal form of p40. This drug is known to inhibit the elongation phase of polypeptide synthesis without much effect on initiation. This accounts for preservation of the polysomal components, even under conditions of poor initiation. It may also be noted that the small p40-bearing particles in the cycloheximide-treated incubation mixture were of the 40S to 60S variety, in contrast to the broader sedimentation range of the particles present in the lysate incubated in the absence of the drug. Cycloheximide did not seem to prevent the release of non-particulate p40. Measurements of total p40 in unfractionated extracts showed that there was no loss of this protein on incubation.

Uptake of p40 into Polysomes in Starved S-180 Cells Exposed to Nutrients. Additional evidence for a functional relationship between p40 and polysome formation was obtained by analysis of S-180 cells exposed to starvation. This treatment causes ribosome runoff and release of the cellular mRNA as nucleoprotein complexes (15). Refeeding of the cells results in rapid polysome assembly (8). p40 in the starved cells occurs primarily as 60S to 80S particles (Fig. 5A) similar to those seen in the incubated reticulocyte lysate (Fig. 4). Within 10 min after addition of nutrients, a large portion of the polysomes have been restored, and a large proportion of the p40 is found in the polysome fraction (Fig. 5B and C). Fig. 5C shows that the sedimentation profile of the p40 transferred to this fraction follows quite closely that of the polysomes.

DISCUSSION

p40 Is a Component of Polyribosomes. The present results describe a cellular component apparently involved in the translation process. This component occurs to a large extent as a heterogeneous population of small cytoplasmic particles that sediment primarily around 40S to 60S. The particles are characterized by the presence of a 33-kDa polypeptide, termed p40. Our belief that p40 is involved in the translation process is based on its association with polyribosomes. A large fraction of the cellular p40 cosediments with these structures (see Figs. 2, 4, and 5). The association seen in sucrose gradients is not due simply to aggregation of the 40S to 60S p40-bearing particles on the polysomal structures during centrifugation. This is indicated by the fact that RNase digestion releases p40 as part of a structure larger than the "free" 40S to 60S particles seen in untreated extracts (Fig. 3). Disruption of polysomes caused by ribosome runoff in intact cells exposed to starvation also leads to loss of the polysomal

FIG. 4. Shifts in distribution of p40 in the reticulocyte lysate in relation to translation. Samples of lysate were incubated for 15 min at 37°C under conditions optimal for translation (11) in the presence (C) or absence (B) of cycloheximide (20 μ g/ml). (A) Mixture before incubation. This was followed by zone centrifugation for 90 min through a 10–30% sucrose gradient having at the bottom 0.25 ml of 2 M sucrose. Position of 60S size marker was obtained by centrifugation of a MEL extract in a separate tube. Fraction 1, p40 present in the pellet as well as in the first fraction.

FIG. 5. Uptake of p40 into polysomes of S-180 cells during recovery from starvation. Cells were incubated in medium devoid of nutrients for 1 h as described (15). Recovery was initiated by addition of glucose, amino acids, and serum; samples were taken before (A) and 10 min after (B and C) refeeding. Cytoplasmic extracts were centrifuged through 5-ml sucrose gradients for 45 min (C) and 90 min (A and B). Amount of recovered cell extract used for longer centrifugation (C) was less than that used for shorter centrifugation (B).

form of p40, with accumulation of p40-containing particles of sizes similar to those released by RNase (compare Figs. 5A and 3B). In the starved cells, refeeding results in rapid polysome reconstitution, with the concomitant appearance of p40 in the newly generated polysomes (Fig. 5). Tight correlation between polysomes and p40 is also seen in the reticulocyte cell-free translation system. The polysomal p40 in the extract is lost when this extract is incubated under conditions that lead to ribosome runoff. This loss is prevented to a large extent when ribosome runoff is blocked by the elongation inhibitor cycloheximide (Fig. 4).

Nature of the Small 40S to 60S Particles Associated with p40.

The sedimentation characteristics of these particles are similar to those of the translation preinitiation complex consisting of the 40S ribosomal subunit associated with initiation factors (16, 17). It is unlikely, however, that they represent some form of the 40S initiation complex to which p40 is bound. Our measurements of cellular p40 levels indicate that there are six to eight molecules of this protein for each ribosome. The 40S to 60S fraction contains about one-third of the cellular p40 but only a small portion of the ribosomal particles. Thus, there must be a very high ratio of p40 to 40S ribosomal subunits in this fraction. If both were present in the same structure, the latter would have to consist of a large number of p40 molecules bound to a single ribosomal particle. It seems more reasonable to conclude that p40 occurs in a structure distinct from the 40S preinitiation complex.

It can also be concluded from our data that the 40S to 60S p40-containing particles are not associated with mRNA or with any other RNA readily accessible to RNase, since the RNase digestion that destroys the polysomal mRNA has no obvious effect on the sedimentation behavior of these particles. It was verified that RNase treatment also destroys the mRNA in free ribonucleoproteins.

The p40-containing material released from polysomes consists of larger particles sedimenting in a relatively heterogeneous fashion around 80S. These are unlikely to represent the 40S to 60S particles associated with mRNA or mRNA fragments. Similar size distributions are seen when the polysomes are disrupted by thorough RNase digestion or by ribosome runoff, which does not involve destruction of the mRNA (compare Figs. 3 and 5). The material derived from polysomes could represent the 40S to 60S particles associated with additional material in a more complex structure or perhaps p40 bound to the 80S ribosomes.

The Soluble Form of p40. MEL cell extracts contained some p40 not associated with particles (see Fig. 2). The amounts of the soluble form tended to be quite variable, with relatively high levels in rapidly growing cells and low levels in cells approaching the stationary phase of growth (data not shown). We have observed that MEL cells induced to differentiate by exposure to dimethyl sulfoxide also have little soluble p40. It may perhaps be significant that the S-180

ascites cells, which have very little soluble p40 (see Figs. 3 and 5), are unable to grow in culture.

Experiments with rabbit reticulocyte extracts, which are also rich in the soluble form of p40 (see Fig. 4), showed that the latter is released from particles when the extract is incubated under conditions that lead to ribosome runoff. It is not clear, however, whether the added soluble p40 was derived primarily from the polysomes or from the smaller particles. It may be significant that inhibition of polypeptide chain elongation by cycloheximide, which tends to preserve the polysomal form of p40, did not prevent release of p40 from the cytoplasmic particles. This behavior suggests that p40 may be cycled between small particles, polysomes, and a soluble form during translation. It seems that the reticulocyte lysate may be deficient in the ability to reincorporate the soluble p40 into a particulate form. In the stationary MEL cells, as well as in the S-180 ascites cells, release of p40 as soluble material seems to be relatively inefficient. The differences in relative amounts of particulate and soluble p40 could be due to variability in rate-limiting steps in this apparent cycling process.

Significance of p40. Our results point to the occurrence of this protein in an as yet uncharacterized cytoplasmic structure that somehow becomes part of the polyribosome. Previous evidence tends to suggest that polysome formation is accompanied by association of the mRNA with a large structure (8). There are also indications that polyribosomes are associated with a cytoskeletal framework (14, 18, 19) and that this kind of association may be essential for mRNA translation in the cell (20). This raises the possibility that the p40-containing particles might be involved in the polysome-cytoskeleton interaction. Immunocytochemical studies have shown association of p40 with stress fibers in 3T3 cells (12), although distribution throughout the cytoplasm as small granules has also been seen (21). The occurrence of p40 in reticulocytes, however, suggests that this protein also functions in translation in the absence of extensive cytoskeleton. Association of components of the translation apparatus with the cytoskeleton is not without precedent. The elongation factor EF-1a has been identified as an actin filament bundling protein (22).

The biological role of p40 remains a matter of conjecture at this point. In view of its wide distribution in nature (12), it must fulfill a basic cell function. Its amino acid sequence appears to be highly conserved, as indicated by the fact that the human and mouse species differ by only two amino acids (4, 5). Some sort of connection with cell proliferation is suggested by the fact that the mRNA for this protein was found to be highly elevated in a human colon carcinoma (5). p40 shows homology to a 67-kDa laminin receptor (6, 7), although the extent of homology is not known. This homology could be fortuitous, or it could reflect some functional relationship between the two proteins. If this were the case,

p40 might provide a link between protein synthesis and cell-surface events. There is also the intriguing possibility that p40 function may be linked to phases of embryonic development, as suggested by cytochemical studies on mammalian embryonic retina (12). The fact that synthesis of this protein is under translational control (2, 3) provides further indication of a regulated function for p40 in the cell.

We are very grateful to Dr. Ursula Drager for the gift of the monoclonal antibody to p40, to Dr. William Merrick for the gift of the p40 protein, and to Dr. Michael Berne of the Physiology Department of Tufts University for the microamino acid analysis of p40. We also thank Mr. Ronald Auth for help in preparation of illustrations for the manuscript. This work was supported by Research Grant GM 17973 from the National Institutes of Health.

1. Geoghegan, T., Cereghini, S. & Brawerman, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5587-5591.
2. Yenofsky, R., Bergmann, I. & Brawerman, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5876-5880.
3. Yenofsky, R., Cereghini, S., Krowczynska, A. & Brawerman, G. (1983) *Mol. Cell. Biol.* **3**, 1197-1203.
4. Makrides, S., Chitpatima, S. T., Bandyopadhyay, R. & Brawerman, G. (1988) *Nucleic Acids Res.* **16**, 2349.
5. Yow, H., Wong, J. M., Chen, H. S., Lee, C., Steele, G. D., Jr., & Chen, L. B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6394-6398.
6. Wewer, U. M., Liotta, L. A., Jaye, M., Ricca, G. A., Drohan, W. N., Claysmith, A. P., Rao, C. N., Wirth, P., Coligan, J. E., Albrechtsen, R., Mudryj, M. & Sobel, M. E. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7137-7141.
7. Rao, C. N., Castronovo, V., Schmitt, M. C., Wewer, U. M., Claysmith, A. P., Liotta, L. A. & Sobel, M. (1989) *Biochemistry* **28**, 7476-7486.
8. Sonenshein, G. E. & Brawerman, G. (1977) *Eur. J. Biochem.* **73**, 307-312.
9. Albrecht, G., Krowczynska, A. & Brawerman, G. (1984) *J. Mol. Biol.* **178**, 881-896.
10. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.
11. Cereghini, S., Geoghegan, T., Bergmann, I. & Brawerman, G. (1979) *Biochemistry* **18**, 3153-3159.
12. Rabacchi, S. A., Neve, R. L. & Drager, U. C. (1990) *Development* **109**, 521-531.
13. Blake, M. S., Johnston, K. H., Russell-Jones, G. J. & Gotschlich, E. C. (1984) *Anal. Biochem.* **136**, 175-179.
14. Lee, S. Y., Krsmanovic, V. & Brawerman, G. (1971) *J. Cell Biol.* **49**, 683-691.
15. Lee, S. Y., Krsmanovic, V. & Brawerman, G. (1971) *Biochemistry* **10**, 895-900.
16. Benne, R. & Hershey, J. W. B. (1978) *J. Biol. Chem.* **253**, 3078-3087.
17. Shafritz, D. A., Weinstein, J. A., Safer, B. S., Merrick, W. C., Weber, L. A., Hickey, E. D. & Baglioni, C. (1976) *Nature (London)* **261**, 291-294.
18. Lenk, R., Ransom, L., Kaufmann, Y. & Penman, S. (1975) *Cell* **10**, 67-78.
19. Howe, J. G. & Hershey, W. B. (1984) *Cell* **37**, 85-93.
20. Cervera, M., Dreyfuss, G. & Penman, S. (1981) *Cell* **23**, 113-120.
21. McCaffery, P., Neve, R. L. & Drager, U. C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8570-8574.
22. Yang, F., Demma, M., Warren, V., Dharmawardhane, S. & Condeelis, J. (1991) *Nature (London)* **347**, 494-496.