Inhibition of protein synthesis alters the subcellular distribution of mRNA in neurons but does not prevent dendritic transport of RNA

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ABSTRACT This study evaluates whether protein synthesis plays a role in targeting RNA molecules to different subcellular domains within neurons. Transport of newly synthesized RNA (labeled with [3H]uridine) was examined in the presence of the protein synthesis inhibitors puromycin and cycloheximide. In situ hybridization was used to determine whether inhibition of protein synthesis altered the subcellular distribution of mRNAs. Transport of recently synthesized RNA was not disrupted after prolonged exposure to either inhibitor. However, inhibition of protein synthesis caused several mRNAs that are normally confined to the cell body to appear in dendrites. The distribution of mRNAs that are normally present in dendrites was unaffected. These findings suggest that protein synthesis is not required to translocate RNA into the dendrites but may play a role in restricting particular mRNAs to the neuronal cell body.

Gene expression in differentiated cells is thought to be regulated in part by the spatial organization of nuclear and cytoplasmic matrices (reviewed in ref. 1). The cytoarchitecture of nuclear and cytoplasmic networks is critical for replication and transcription of DNA as well as translation of RNA (2–5). RNA synthesis occurs in defined domains of the nucleus, and there appears to be a conduit system for exporting newly synthesized RNA to the cytoplasm (6–9). Once mRNA emerges in the cytoplasm it is often targeted to a particular subcellular destination prior to translation (10–16), which offers an additional level of regulation over cellular gene expression.

One of the most dramatic examples of selective mRNA localization occurs in neurons. Most neuronal mRNAs are found only in neuronal cell bodies (17, 18). In contrast, a few mRNAs including the mRNAs encoding microtubule-associated protein 2 (MAP2) and the 2 subunit of calcium/calmodulin-dependent protein kinase II and the polymerase III transcript BCI are found in dendrites at considerable distance from the nucleus (10, 11, 17–20). In fact, MAP2 mRNA can be detected >100 μm away from the cell body in cultured neurons (18). This differential distribution implies the existence of sorting signals that direct some mRNAs to the dendrites and retain others in the cell body. A mechanism for transporting RNA into dendrites has been demonstrated by pulse-labeling newly synthesized RNA with [3H]uridine and then following the migration of labeled RNA into the dendrites (21, 22). However, little is known about this transport machinery and the signals it may utilize except that some of the newly synthesized RNA transported to the dendrites is attached to the cytoskeleton (21).

The process that delivers RNA to dendrites must select for a subpopulation of mRNAs. One means by which the dendritic transport machinery may recognize RNAs targeted for transport is via interaction with nascent polypeptides undergoing synthesis. This notion is based on analogy with the mechanism that targets mRNAs encoding membrane proteins to the rough endoplasmic reticulum. The N-terminal domain of the nascent polypeptide is recognized by a signal recognition particle (reviewed in ref. 23). The signal recognition particle prevents translation until the complex docks with an appropriate receptor in the endoplasmic reticulum. In a similar way, nascent peptides could target particular mRNAs to the dendritic transport machinery.

Other studies have considered whether this sort of mechanism operates in other cell types. For example, Sundell and Singer (24) have shown that blocking protein synthesis does not disrupt actin mRNA localization in migrating fibroblasts. In the present study, we have used a similar approach to determine whether protein synthesis is required for dendritic RNA transport in neurons. If mRNA is targeted to the dendritic transport machinery by nascent peptides, then dendritic transport should be disrupted by treatments that inhibit protein synthesis. Puromycin and cycloheximide were used to assess the effect of protein synthesis inhibition on the transport of recently synthesized RNA into dendrites. We also examined whether the inhibitors disrupt the normal pattern of mRNA compartmentation by examining the distribution of particular mRNAs with in situ hybridization.

Our results reveal that newly synthesized RNA continues to move into the dendrites when protein synthesis is blocked and the inhibition of protein synthesis for short (2 hr) periods does not alter the subcellular localization of particular mRNAs. To our surprise, prolonged blockade of protein synthesis disrupts RNA compartmentation, so that mRNAs normally confined to the cell body move into the dendrites.

MATERIALS AND METHODS

Cell Cultures. Cultures of hippocampal neurons were prepared as described (25, 26). Neurons were plated onto polylysine-treated glass coverslips (Assistent Brand; Carolina Biological Supply) at a density of 50,000 cells per 60-mm dish. Experiments were carried out on neurons that had been maintained in culture for 2 weeks.

Protein Synthesis Inhibitors. Cycloheximide and puromycin were stored as stock solutions of 20 mg/ml and 25 mg/ml, respectively, in dimethyl sulfoxide (DMSO). The inhibitors were added to the culture medium to obtain final concentrations of 20 μg/ml (cycloheximide) and 200 μg/ml (puromycin). The effect of the inhibitors on protein synthesis was evaluated by scintillation counting. Neurons were treated with inhibitors for 1 hr and incubated with [3H]leucine (38 μCi per ml of medium; 1 Ci = 37 GBq) for 30 min. Coverslips with adherent neurons were fixed as described below and crushed with forceps. Quantitative evaluation of protein synthesis by

Abbreviations: DMSO, dimethyl sulfoxide; MAP2, microtubule-associated protein 2; GAP-43, growth-associated protein 43; cRNA, complementary RNA.

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scintillation counting revealed that puromycin and cycloheximide inhibited \(^{3}H\)leucine incorporation by about 95% and 91%, respectively (data not shown). To determine whether neurons were metabolically active after prolonged exposure to protein synthesis inhibitors, cells exposed to inhibitor for 1, 6, 12, or 24 hr were pulse-labeled with \(^{3}H\)uridine for 1 hr and then fixed, dehydrated, and prepared for autoradiography or counted in a scintillation counter.

**Pulse Labeling of RNA.** Preliminary studies were undertaken to evaluate possible experimental strategies for assessing the role of protein synthesis in the transport of newly synthesized RNA. Neurons treated with cycloheximide or puromycin either before or after a 1-hr incubation in \(^{3}H\)uridine at 37°C continued to transport RNA into dendrites (data not shown). However, addition of inhibitors after the pulse might not completely prevent the synthesis of polypeptides from the labeled RNAs and addition of inhibitors during the period of pulse labeling might alter the rate of \(^{3}H\)uridine incorporation. In fact, in preliminary experiments cycloheximide-stimulated incorporation of \(^{3}H\)uridine, consistent with the previous findings of Ch'ih et al. (27, 28).

To avoid these problems we used an alternative approach that took advantage of the fact that nucleocytoplasmic transport of newly synthesized RNA is blocked at room temperature and can be reinstated when cells are warmed to 37°C (29). Neurons were pulse-labeled with \(^{3}H\)uridine (50 μCi/ml) at 25°C for 1 hr. Labeling neurons at room temperature will result in less RNA synthesis than would labeling at 37°C. Nevertheless, because the same temperature and conditions are used for the control neurons, this should not affect the interpretation of the outcome. After the labeling period, puromycin or cycloheximide plus an excess of unlabeled uridine was added. Neurons were maintained at 25°C for an additional 3 hr to allow maximal inhibition of protein synthesis while retaining the previously labeled RNA in the nucleus. The neurons were then warmed to 37°C and maintained for up to 8 hr. Control neurons were pulse-labeled at 25°C, maintained at 25°C for 3 hr, and then returned to 37°C in the absence of inhibitor for the same chase interval.

**Fixation.** Neurons were fixed for 10-15 min in 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS) warmed to 37°C. Neurons were then dehydrated in a graded series of ethanol and air dried.

**Autoradiography.** Coverslips were mounted on clean slides, dipped in Kodak NTB2 autoradiography emulsion, and exposed in the dark for 6 days. Autoradiographs were developed with Kodak D-19 and slides were coverslipped with Kässer’s glycerol jelly.

**Hybridization of Cultured Neurons with Complementary RNA (cRNA) Probes.** Neurons prepared for *in situ* hybridization were treated with cycloheximide (20 μg/ml) or puromycin (200 μg/ml) or 0.08% DMSO for 2, 4, 6, 8, 12, or 24 hr before fixation. It should be pointed out that neurons treated with protein synthesis inhibitors and then prepared for *in situ* hybridization did not undergo any room temperature incubations. Fixed cells were rinsed in PBS and stored in 70% ethanol at 4°C until hybridization. The cRNA probes have been described (18) except for the probe for β-actin. The cDNA for the MAP2 probe was a gift from A. Matus (clone 19a; ref. 11). The chicken β-tubulin and β-actin cDNAs were gifts from M. Kirschner (30). The growth-associated protein 43 (GAP-43) cDNA was a gift from A. Rottenberg (31). The chicken β-actin cDNA clone is complementary to the entire 2.0-kb coding region. The cDNA was subcloned into pBR322. This plasmid was linearized with BamHI and transcribed with the T7 promoter to produce full-length antisense probes.

All cRNA probes were labeled by transcription reactions in the presence of uridine 5'-[α-\(^{32}P\)]thiotriphosphate, using the RNA transcription kit from Stratagene (T3 or T7). SP6 enzyme was purchased from Promega. The specific activity of the antisense probes ranged from 3.1 to 5.6 × 10⁶ cpm per μg of RNA. Neurons were hybridized with cRNA probes as described (18).

**Quantitative Assessment of the Distance of Dendritic Labeling.** The distance of dendritic labeling was assessed in a sample of 24-27 neurons from each condition. Individual neurons were selected on the basis of predetermined stage coordinates and photographed in dark field. Photographic prints were made at a final magnification of ×370. The end of dendritic labeling was considered the farthest distance at which a 10-μm circular template over the dendrite contained less than three silver grains. The distance between the center of the cell body and the end of dendritic label was measured with Bio-Quant software, and the average distance of dendritic labeling for each neuron was determined. Student’s *t* tests were used to compare the average distance of dendritic labeling in each group. To determine how far GAP-43 mRNA extended in to the dendrites, 29 neurons were selected for measurements. Neurons were included in the sample only if they displayed considerable labeling of the dendrites.

**RESULTS**

**Transport of Newly Synthesized RNA in the Presence of Protein Synthesis Inhibitors.** To examine the transport of newly synthesized RNA, neurons were fixed at different times after pulse labeling with \(^{3}H\)uridine and the migration of recently synthesized RNA from the nucleus into the somatodendritic domain was examined by autoradiography. \(^{3}H\)Ur dine labels rRNA, mRNA, and tRNA, but under the fixation condition used here unincorporated uridine and tRNA would not be retained (21, 32). Thus, the labeling of newly synthesized RNA into the dendrites was evidenced in control neurons by the migration of labeled RNA from the nucleus into dendrites over the 6-hr chase interval (compare Fig. 1A and B). Translocation also occurred in neurons treated with cycloheximide or puromycin (Fig. 1C and D). The average distance of dendritic labeling in cycloheximide-treated neurons was 71 μm (n = 26), compared with 79 μm in matched controls (6-hr chase). These values were not significantly different (*P* = 0.162). The average distance of dendritic labeling in puromycin-treated neurons was 98 μm (n = 26), which was somewhat greater than in matched control neurons (82 μm; *P* = 0.004). Thus, neither inhibitor interfered with translocation of newly synthesized RNA into dendrites.

**Intracellular Distribution of mRNAs in the Presence of Protein Synthesis Inhibitors.** Since \(^{3}H\)uridine labeling of newly synthesized RNA cannot differentiate between movement of rRNA and mRNA, a different approach was used to examine the effect of protein synthesis inhibitors on the distribution of particular mRNAs. Neurons treated with cycloheximide or puromycin for periods ranging from 2 hr to 20 hr were prepared for hybridization with cRNA probes to mRNAs encoding GAP-43, actin, and tubulin. Neurons incubated without inhibitor exhibited a normal mRNA distribution; GAP-43, actin, and tubulin mRNAs were restricted to the cell body (Fig. 2A, D, and G). However, treatment of neurons with either cycloheximide or puromycin for 20 hr resulted in a profound change in mRNA distribution. mRNAs that are normally restricted to the cell body, including those encoding GAP-43 (Fig. 2A–C), tubulin (Fig. 2D–F), and actin (Fig. 2G–I), (Fig. 2G–I) appeared in the dendrites. In many instances, the label extended over all of the primary dendrites. In some cases, only some of the dendrites were labeled, while in other cases the label still appeared to be restricted to the cell body. After 20 hr of treatment with either puromycin or cycloheximide, MAP2 mRNA was still detectable in cell bodies and dendrites of most neurons (Fig. 3), but
the overall level of labeling appeared to be substantially reduced after inhibitor treatment.

To determine how quickly mRNAs that were normally restricted to the cell body entered the dendrites, the distribution of GAP-43 mRNA was examined 2–24 hr after inhibitor treatment. No dendritic labeling was observed after 2 hr, slight labeling of proximal dendrites was seen after 4 hr, and prominent labeling was visible by 6 hr (Fig. 4). The farthest distance of dendritic labeling measured in neurons treated for 6 hr was on average 81 ± 15 μm (n = 29 neurons). Longer exposure to protein synthesis inhibitors, for 8, 12, or 24 hr, caused no further change in mRNA distribution, although the overall levels of labeling decreased with prolonged inhibitor treatment. The time course of mRNA redistribution after treatment with cycloheximide was similar to that produced by puromycin, but the amount of dendritic labeling was consistently less.

Inhibition of protein synthesis for prolonged periods can be toxic. A few dead cells, readily identifiable by phase-contrast microscopy, were apparent after 12 hr of drug treatment and their numbers increased after 24 hr. Treatment for shorter periods had little obvious effect on neuronal survival. More important, neurons treated with protein synthesis inhibitors remained metabolically active, as evidenced by their ability to synthesize RNA. Scintillation counts of cultures that had been pulse-labeled with [3H]uridine indicated that RNA synthesis continued even after inhibition of protein synthesis for 24 hr although the overall level of incorporation decreased somewhat with time in the inhibitor. When cultures were treated with inhibitors for 6 or 12 hr and then pulse-labeled with [3H]uridine and analyzed by autoradiography, the majority of neurons still incorporated [3H]uridine. Thus, the alteration of mRNA distribution evident after exposure to protein synthesis inhibitors for 6 or 12 hr is not likely to be simply due to a reduction in neuronal viability.

**DISCUSSION**

The mechanisms that permit neurons to target particular mRNAs to specific subcellular destinations are unknown. The experiments reported here represent an attempt to define some of the properties of the sorting and transport mechanisms. Our results show that translocation of newly synthe-
sized RNA into dendrites continues in the presence of protein synthesis inhibitors. However, this treatment caused a profound change in the distribution of several mRNAs. After 4–6 hr of exposure to protein synthesis inhibitors, actin, tubulin, and GAP-43 mRNAs, which are normally restricted to the cell body, migrate into the dendrites. The distribution of MAP2 mRNA, which is normally dendritic, is unaffected.

The initial intent of this study was to determine whether the targeting and transport of RNAs into dendrites depend on a signal sequence-like mechanism. That the translocation of RNA labeled with [3H]uridine persists in neurons treated with protein synthesis inhibitors suggests that this process does not require ongoing message translation. Taken at face value, this would rule out a targeting mechanism based on the nascent peptide. However, this interpretation is based on the assumption that the mechanism of RNA translocation in control and drug-treated neurons is identical. It is conceivable that inhibition of protein synthesis blocks normal dendritic transport but that RNA translocation then occurs via a different process, such as diffusion. In most cells, RNA is attached to the cytoskeleton (4) and is apparently unable to diffuse over significant distances (33–35). Puromycin causes release of the nascent polypeptide and dissociation of the polyribosome (36). In fibroblasts, polyribosome dissociation results in release of a large proportion of the total RNA from the cytoskeleton, although most mRNAs remain anchored (36). Such an effect could permit the diffusion of newly synthesized non-messenger RNAs, an effect that might resemble normal dendritic RNA transport. If this were the case, cycloheximide, which stabilizes polyribosomes (37–39), might be expected to have an effect opposite that of puromycin. Like puromycin, cycloheximide would interfere with a signal sequence-mediated transport but would not be expected to enhance RNA diffusion. In fact, RNA translocation continues normally in the presence of cycloheximide. Given these considerations, and the fact that the rate of RNA translocation is similar in control and inhibitor-treated neurons, it seems most likely that the RNA translocation observed in the presence of protein synthesis inhibitors occurs via the same mechanisms as that in control cells.

The redistribution of those mRNAs normally confined to the cell body that was induced by protein synthesis inhibitors was quite unexpected. This redistribution of mRNA could have occurred in any of at least three different ways. Inhibition of protein synthesis could (i) cause the release of mRNAs that are normally anchored within the cell body, and so are prevented from entering dendrites; (ii) interfere with the selectivity of the dendritic transport machinery so that mRNAs normally restricted to the cell body are mistakenly transported; or (iii) increase the half-life of mRNAs generally, thereby extending the distribution of messages that normally are degraded before they reach the dendrites. Of these possibilities, the last seems the least likely. Cycloheximide treatment is known to stabilize some mRNAs (40, 41), but puromycin has not been reported to have this effect (41). Beyond this, a large number of different mRNAs (probably with very different half-lives) are restricted to the cell body; it seems somewhat unlikely that the only mechanism restricting mRNAs to the cell body depends on their having a short half-life. Our results are compatible with either of the other two possibilities.

How might inhibition of protein synthesis interfere with the anchoring or targeting of neuronal mRNAs? One possibility is that the restriction of mRNAs to the cell body—but not

FIG. 3. Neurons maintained in protein synthesis inhibitors for 20 hr exhibit dendritic labeling with probes to MAP2 mRNA. Dark-field photomicrographs showing distribution of MAP2 mRNA as revealed by in situ hybridization using 35S-labeled cRNA probes. (A) Distribution of MAP2 mRNA in control neurons. (B and C) Distribution of MAP2 mRNA in neurons that had been maintained in cycloheximide (B) or puromycin (C) for 20 hr. (Bar = 50 μm.)

FIG. 4. Time course of appearance of GAP-43 mRNA in dendrites after treatment with puromycin. Dark-field photomicrographs showing distribution of GAP-43 mRNA as revealed by in situ hybridization using 35S-labeled cRNA probes. (A) Distribution of GAP-43 mRNA in control neurons. (B–F) Distribution of GAP-43 mRNA after treatment with puromycin (200 μg/ml) for 2 hr (B), 4 hr (C), 6 hr (D), 8 hr (E), or 24 hr (F). (Bar = 40 μm.)
their transport into the dendrites—depends on a signal sequence mechanism. A related possibility is that mRNAs are retained in the cell body by a number of different translation-dependent processes and that all mRNAs that are not being translated are transported. For example, actin and tubulin are assembled into cytoskeletal elements as they are being synthesized (so-called cotranslational assembly; ref. 42). Both puromycin and cycloheximide could disrupt anchoring based on cotranslational assembly. The mRNAs for membrane proteins would presumably be anchored to the endoplasmic reticulum via a signal sequence mechanism (which would be disrupted by the inhibitors). And mRNAs for other proteins (like GAP-43) might be anchored by other translation-dependent processes.

A final possibility is that a relatively short-lived protein(s) plays a role in anchoring somatic mRNAs or preventing them from interacting with the dendritic transport machinery. Prolonged inhibition of protein synthesis may result in turnover of these proteins and consequent redistribution of mRNAs.

Regardless of the mechanisms that cause the mRNAs encoding actin, tubulin, and GAP-43 to move into dendrites after protein synthesis inhibition, it is significant that they are able to leave the cell body at all. In the past, selective transport and targeting of RNA in neurons have been considered primarily in terms of how particular mRNAs are targeted to and translocated within dendrites (21, 22, 43). The present results suggest that the cellular mechanisms responsible for restricting particular mRNAs to the cell body may be as important for mRNA compartmentation in neurons as the mechanisms responsible for the selective delivery of mRNAs into dendrites.

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